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<b>(21) International Application Number:</b> PCT/US92/08999 <b>(22) International Filing Date:</b> 23 October 1992 (23.10.92)  <b>(30) Priority data:</b> 781,356                      25 October 1991 (25.10.91)      US 863,641                      3 April 1992 (03.04.92)                      US  <b>(71) Applicant:</b> SAN DIEGO REGIONAL CANCER CENTER [US/US]; 3099 Science Park Road, Suite 200, San Diego, CA 92121 (US).  <b>(72) Inventors:</b> SOBOL, Robert, E. ; 5673 La Jolla Hermosa Avenue, La Jolla, CA 92037 (US). FRED, H., Gage ; 8388 Caminito Helecho, La Jolla, CA 92037 (US). ROYSTON, Ivor ; 1515 El Camino del Teatro, La Jolla, CA 92037 (US). FRIEDMAN, Theodore ; 9470 La Jolla Shores Drive, La Jolla, CA 92037 (US). FAKHRAI, Habib ; 1538 Avenida Andante, Oceanside, CA 92056 (US).		<b>(74) Agents:</b> CAMPBELL, Cathryn et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US).  <b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> LYMPHOKINE GENE THERAPY OF CANCER  <b>(57) Abstract</b>  A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen product and to secrete at least one cytokine gene product are utilized in a formulation to immunize the patient at a site other than an active tumor site.		

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Lymphokine Gene Therapy of CancerBACKGROUND

This application is a continuation-in-part of United States Patent Application Serial No. 07/781,356, filed on October 25, 1991, which is a continuation-in-part of United States Patent Application Serial No. 07/720,872, filed on June 25, 1991, both of which are incorporated herein in their entirety.

Recent advances in our understanding of the biology of the immune system have lead to the identification of important modulators of immune responses, called cytokines (1-3). Immune system modulators produced by lymphocytes are termed lymphokines, a subset of the cytokines. These agents mediate many of the immune responses involved in anti-tumor immunity. Several of these cytokines have been produced by recombinant DNA methodology and evaluated for their anti-tumor effects. The administration of lymphokines and related immunomodulators has resulted in objective tumor responses in patients with various types of neoplasms (4-7). However, current modes of cytokine administration are frequently associated with toxicities that limit the therapeutic value of these agents.

For example, interleukin-2 (IL-2) is an important lymphokine in the generation of anti-tumor immunity (4). In response to tumor antigens, a subset of lymphocytes termed helper T-cells secrete small quantities of IL-2. This IL-2 acts locally at the site of tumor antigen stimulation to activate cytotoxic T-cells and natural killer cells which mediate systemic tumor cell destruction. Intravenous, intralymphatic and intralesional administration of IL-2 has resulted in clinically significant responses in some cancer patients (4-6). However, severe toxicities (hypotension and adema) limit the dose and efficacy of intravenous and intralymphatic IL-

2 administration (5-7). The toxicity of systemically administered lymphokines is not surprising as these agents mediate local cellular interactions and they are normally secreted in only very small quantities.

5                    Additionally, other cytokines, such as interleukin-4 (IL-4), alpha interferon ( $\alpha$ -INF) and gamma interferon ( $\gamma$ -INF) have been used to stimulate immune responses to tumor cells. Like IL-2, the current modes of administration have adverse side effects.

10                    To circumvent the toxicity of systemic cytokine administration, several investigators have examined intralesional injection of IL-2. This approach eliminates the toxicity associated with systemic IL-2 administration (8,9,10). However, multiple intralesional injections are  
15 required to optimize therapeutic efficacy (9,10). Hence, these injections are impractical for many patients, particularly when tumor sites are not accessible for injection without potential morbidity.

                    An alternative approach, involving cytokine gene  
20 transfer into tumor cells, has resulted in significant anti-tumor immune responses in several animal tumor models (11-14). In these studies, the expression of cytokine gene products following cytokine gene transfer into tumor cells has abrogated the tumorigenicity of the cytokine-secreting  
25 tumor cells when implanted into syngeneic hosts. The transfer of genes for IL-2 (11,12)  $\gamma$ -INF (13) or interleukin-4 (IL-4) (14) significantly reduced or eliminated the growth of several different histological types of murine tumors. In the studies employing IL-2 gene  
30 transfer, the treated animals also developed systemic anti-tumor immunity and were protected against subsequent tumor challenges with the unmodified parental tumor (11,12). Similar inhibition of tumor growth and protective immunity was also demonstrated when immunizations were performed

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with a mixture of unmodified parental tumor cells and genetically modified tumor cells engineered to express the IL-2 gene. No toxicity associates with localized lymphokine transgene expression was reported in these  
5 animal tumor studies (11-14).

While the above gene-transfer procedure has been shown to provide anti-tumor immunity, it still retains practical difficulties. This approach is limited by the inability to transfer functional cytokine genes into many  
10 patients' tumor cells, as most patients' tumors cannot be established to grown in vitro and methods for human in vivo gene transfer are not available.

#### SUMMARY OF THE INVENTION

The present invention demonstrates a novel, more  
15 practical method of cytokine cancer immunotherapy. In one approach, selected cells from a patient, such as fibroblasts, obtained, for example, from a routine skin biopsy, are genetically modified to express one or more cytokines. Alternatively, patient cells which may normally  
20 serve as antigen presenting cells in the immune system such as macrophages, monocytes, and lymphocytes may also be genetically modified to express one or more cytokines. These modified cells are hereafter called cytokine-expressing cells, ore CE cells. The CE cells are then  
25 mixed with the patient's tumor antigens, for example in the form of irradiated tumor cells, or alternatively in the form of purified natural or recombinant tumor antigen, and employed in immunizations, for example subcutaneously, to induce systemic anti-tumor immunity.

30 The cytokines are locally expressed at levels sufficient to induce or augment systemic anti-tumor immune responses via local immunization at sites other than active tumor sites. Systemic toxicity related to cytokine

administration should not occur because the levels of cytokin secr ted by the CE cells should not significantly affect systemic cytokine concentrations.

As the amount of cytokine secreted by the CE  
5 cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity, this approach provides the benefit of local cytokine administration. In addition, this novel method obviates the need for intralesional injections, which may produce  
10 morbidity. Furthermore, the continuous local expression of cytokine(s) at the sites of immunization may also augment anti-tumor immune responses compared to intermittent cytokine injections. This method also provides the advantage of local immunization with the CE cells, as  
15 opposed to cumbersome intravenous infusions. This method also eliminates the need for establishing tumor cell lines in vitro as well as transfer of genes into these tumor cells.

This invention also provides an alternative means  
20 of localized expression of cytokines to induce and/or increase immune responses to a patient's tumor through genetic modification of cellular expression of both cytokine(s) and tumor antigen(s). In this embodiment, selected cells from a patient are isolated and transduced  
25 with cytokine gene(s) as well as gene(s) coding for tumor antigen(s). The transduced cells are called "carrier cells." Carrier cells can include fibroblasts and cells which may normally serve as antigen presenting cells in the immune system such as macrophages, monocytes, and  
30 lymphocytes. Transduced carrier cells actively expressing both the cytokine(s) and the tumor antigen(s) are selected and utilized in local immunizations at a site other than active tumor sites to induce anti-tumor immune responses. As with the CE cells, these carrier cells should not  
35 produce substantial systemic toxicities, as the lev ls of

cytokine(s) secreted by the carrier cells should not significantly affect systemic cytokine concentrations. This alternate embodiment is advantageous because it obviates the need to obtain samples of the tumor, which is sometimes difficult. However, carrier cells can be utilized in local immunizations in conjunction with tumor cells, tumor cell homogenates, purified tumor antigens, or recombinant tumor antigens to enhance anti-tumor immunity.

Additionally, this second embodiment retains the same advantages as the first embodiment in that the level of cytokine released by the carrier cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity. In addition, as with the first embodiment, this method obviates the need for intralesional injections, and allows for continuous expression of cytokine(s). This method also eliminates the need for establishing continuous cultures in vitro of tumor cells as well as transfer of genes into these tumor cells, and provides the advantage of local immunization with the carrier cells, as opposed to cumbersome lengthy intravenous infusions.

These approaches may also find application in inducing or augmenting immune responses to other antigens of clinical significance in other areas of medical practice.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic diagrams of retroviral vectors DC/TKIL2, LXSIL-IL2, and LNCX-IL2.

Figure 2 shows a mean IL-2 concentration of triplicate supernatant samples measured by ELISA. Supernatants were harvested from overnight cultures of approximately  $1.5 \times 10^6$  semi-confluent fibroblasts.

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Figure 3 shows biological activity of the IL-2 secreted by the transduced fibroblasts was demonstrated by measuring mean  $^3\text{H}$ -TdR incorporation of an IL-2 dependent T-cell line incubated with triplicate samples of supernatants. Supernatants were harvested from overnight cultures of approximately  $1.5 \times 10^6$  semi-confluent fibroblasts.

Figure 4 shows comparisons between animals injected with  $10^5$  CT26 tumor cells alone ( $\square$ );  $10^5$  CT26 tumor cells and  $2 \times 10^6$  unmodified BALB/C fibroblasts ( $\blacksquare$ );  $10^5$  CT26 tumor cells and  $2 \times 10^6$  IL-2 transduced BALB/C fibroblasts ( $\bullet$ ); and  $10^5$  CT26 tumor cells and  $1 \times 10^6$  transduced BALB/C fibroblasts ( $\circ$ ). Tumor measurements are the mean products of the cross-sectional diameter of the tumors from four animals in each treatment group. The (\*) indicates statistically significant difference ( $P < 0.05$ ) in tumor growth curves.

Figure 5 shows PCR analysis of neomycin phosphotransferase DNA sequences. Lane 1 - positive control pLXSN-RI-IL2. Lanes 2 through 4 tests genomic DNA; Lanes 5 and 6 ovary genomic DNA; Lane 7 negative control, no DNA. Identical results were obtained with liver, spleen and lung genomic DNA (data not shown).

Figure 6 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using  $2 \times 10^6$  fibroblasts mixed with  $5 \times 10^4$  CT26 tumor cells concentrating on the rate of tumor growth.

Figure 7 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using  $2 \times 10^6$  fibroblasts mixed with  $5 \times 10^4$  CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

Figur 8 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using  $2 \times 10^6$  fibroblasts mixed with  $1 \times 10^5$  CT26 tumor cells concentrating on the rate of tumor growth.

5 Figure 9 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using  $2 \times 10^6$  fibroblasts mixed with  $1 \times 10^5$  CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

10 Figure 10 shows the effect of IL-2 modified cells on tumor establishment and development using  $2 \times 10^6$  DCTK-IL2-modified CT26 tumor cells mixed with  $1 \times 10^5$  unmodified CT26 compared to  $2 \times 10^6$  DCTK-IL2-modified fibroblasts mixed with  $1 \times 10^5$  CT26 concentrating on the rate of tumor growth.

15 Figure 11 shows the effect of IL-2 modified cells on tumor establishment and development using  $2 \times 10^6$  DCTK-IL2-modified CT26 tumor cells mixed with  $1 \times 10^5$  unmodified CT26 compared to  $2 \times 10^6$  DCTK-IL2-modified fibroblasts mixed with  $1 \times 10^5$  CT26 concentrating on the time of tumor onset  
20 for the individual animal in each treatment group.

Figure 12 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor  
25 cells 7 days prior to challenge with  $5 \times 10^4$  fresh tumor cells.

Figure 13 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in  
30 each treatment group. Mice were immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor cells 7 days prior to challenge with  $5 \times 10^4$  fr sh tumor cells.

Figure 14 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor cells 14 days prior to challenge with  $5 \times 10^4$  fresh tumor cells.

Figure 15 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor cells 14 days prior to challenge with  $5 \times 10^4$  fresh tumor cells.

#### DETAILED DESCRIPTION

A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. "Gene" is defined herein to be a nucleotide sequence encoding the desired protein. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen gene product and to secrete at least one cytokine gene product are utilized in formulation to immunize the patient at a site other than an active tumor site. Cytokines are preferably expressed in cells which efficiently secrete these proteins into the surrounding milieu. fibroblasts are an example of such cells. Fibroblasts or other cells can be genetically modified to express and secrete one or more cytokines, as described later in this specification.

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Tumor antigens can be provided by several methods, including, but not limited to the following: 1) CE cells can be transduced with gene(s) coding for tumor antigens. These "carrier cells" are then utilized in patient immunizations. 2) Cloned gene sequences coding for appropriate tumor antigens can be transferred into cells such as fibroblasts or antigen-presenting cells. These cells are then mixed with CE or carrier cells to immunize the patient. 3) Tumor antigens can be cloned in bacteria or other types of cells by recombinant procedures. These antigens are then purified and employed in an immunization with CE and/or carrier cells. 4) Tumor antigens can be purified from tumor cells and used, along with CE or carrier cells, to immunize the patient. 5) Tumor cells may be irradiated or mechanically disrupted and mixed with CE and/or carrier cells for patient immunizations.

This invention encompasses the following steps: (A) isolation of appropriate cells for generation of CE cells or carrier cells; (B) isolation of cytokine genes or isolation of cytokine genes and tumor antigen genes, as well as appropriate marker and/or suicide genes; (C) transfer of the genes from (B) to produce the CE cells or carrier cells; (D) preparation of immunological samples of the patient's tumor antigens or other suitable tumor antigens for immunization with CE or carrier cells; (E) inactivation of the malignant potential of tumor cells if they are used as a source of tumor antigens for immunization; and (F) preparation of samples for immunization. Following are several embodiments contemplated by the inventors. However, it is understood that any means known by those in the art to accomplish these steps will be usable in this invention.

(A) Isolation of Cells to Generate CE and Carrier Cells

Cells to be utilized as CE cells and carrier cells can be selected from a variety of locations in the patient's body. For example, skin punch biopsies provide a readily available source of fibroblasts for use in generating CE cells, with a minimal amount of intrusion to the patient. alternatively, these fibroblasts can be obtained from the tumor sample itself. Cells of hematopoietic origin may be obtained by venipuncture, bone marrow aspiration, lymph node biopsies, or from tumor samples. Other appropriate cells for the generation of CE or carrier cells can be isolated by means known in the art. Non-autologous cells similarly selected and processed can also be used.

(B) Isolation of Genes

Numerous cytokine genes have been cloned and are available for use in this protocol. The genes for IL-2,  $\gamma$ -INF and other cytokines are readily available (1-5, 11-14). Cloned genes of the appropriate tumor antigens are isolated according to means known in the art.

Selectable marker genes such as neomycin resistance (Neo<sup>R</sup>) are readily available. Incorporation of a selectable marker gene(s) allows for the selection of cells that have successfully received and express the desired genes. Other selectable markers known to those in the art of gene transfer may also be utilized to generate CE cells or carrier cells expressing the desired transgenes.

"Suicide" genes can be incorporated into the CE cells or carrier cells to allow for selective inducible killing after stimulation of the immune response. A gene

such as the herpes simplex virus thymidine kinase gene (TK) can be used to create an inducible destruction of the CE cells or carrier cells. When the CE cells or carrier cells are no longer useful, a drug such as acyclovir or gancyclovir can be administered. Either of these drugs will selectively kill cells expressing TK, thus eliminating the implanted transduced cells. Additionally, a suicide gene may be a gene coding for a non-secreted cytotoxic polypeptide attached to an inducible promoter. When destruction of the CE or carrier cells is desired, the appropriate inducer of the promoter is administered so that the suicide gene is induced to produce cytotoxic polypeptide which subsequently kills the CE or carrier cell. However, destruction of the CE or carrier cells may not be required.

Genes coding for tumor antigen(s) of interest can be cloned by recombinant methods. The coding sequence of an antigen expressed by multiple tumors may be utilized for many individual patients.

20 (C) Transfer of Genes

Numerous methods are available for transferring genes into cultured cells (15). For example, the appropriate genes can be inserted into vectors such as plasmids or retroviruses and transferred into the cells. Electroporation, lipofection and a variety of other methods are known in the field and can be implemented.

One method for gene transfer is a method similar to that employed in previous human gene transfer studies, where tumor infiltrating lymphocytes (TILs) were modified by retroviral gene transduction and administered to cancer patients (16). In this Phase I safety study of retroviral mediated gene transfer, TILs were genetically modified to express the Neomycin resistance (Neo<sup>R</sup>) gene. Following

intravenous infusion, polymerase chain reaction analyses consistently found genetically modified cells in the circulation for as long as two months after administration. No infectious retroviruses were identified in these  
5 patients and no side effects due to gene transfer were noted in any patients (16). These retroviral vectors have been altered to prevent viral replication by the deletion of viral gag, pol and env genes.

When retroviruses are used for gene transfer,  
10 replication competent retroviruses may theoretically develop by recombination between the retroviral vector and viral gene sequences in the packaging cell line utilized to produce the retroviral vector. We will use packaging cell lines in which the production of replication competent  
15 virus by recombination has been reduced or eliminated. Hence, all retroviral vector supernatants used to infect patient cells will be screened for replication competent virus by standard assays such as PCR and reverse transcriptase assays (16). Furthermore, exposure to  
20 replication competent virus may not be harmful. In studies of subhuman primates injected with a large inoculum of replication competent murine retrovirus, the retrovirus was cleared by the primate immune system (17). No clinical illnesses or sequelae resulting from replication competent  
25 virus have been observed three years after exposure. In summary, it is not expected that patients will be exposed to replication competent murine retrovirus and it appears that such exposure may not be deleterious (17).

30 (D) Preparation of Immunological Samples of the Patient's Tumor Antigens or Purified Recombinant Tumor Antigens

Tumor cells bearing tumor associated antigens are isolated from the patient. These cells can derive either from solid tumors or from leukemic tumors. For solid

tumors, single cell suspensions can be made by mechanical separation and washing of biopsy tissue (18).

Hematopoietic tumors may be isolated from peripheral blood or bone marrow by standard methods (19).

5           A second variant is the use of homogenates of tumor cells. Such homogenates would contain tumor antigens available for recognition by the patient's immune system upon stimulation by this invention. Either unfractionated cell homogenates, made, for example, by mechanical  
10 disruption or by freezing and thawing the cells, or fractions of homogenates preferably with concentrated levels of tumor antigens, can be used.

          Likewise, purified tumor antigens, obtained for example by immunoprecipitation or recombinant DNA methods,  
15 could be used. Purified antigens would then be utilized for immunizations together with the CE cells and/or carrier cells described above to induce or enhance the patient's immune response to these antigens.

          In the embodiments employing carrier cells, tumor  
20 antigens are available through their expression by the carrier cells. These carrier cells can be injected alone or in conjunction with other tumor antigen preparations or CE cells. Likewise, when CE cells are used, purified recombinant tumor antigen, produced by methods known in the  
25 art, can be used.

          If autologous tumor cells are not readily available, heterologous tumor cells, their homogenates, their purified antigens, or carrier cells expressing such antigens could be used.

(E) Inactivation of Tumor Cells

When viable tumor cells are utilized in immunizations as a source of tumor antigens, the tumor cells can be inactivated so that they do not grow in the patient. Inactivation can be accomplished by several methods. the cells can be irradiated prior to immunization (18). This irradiation will be at a level which will prevent their replication. Such viable calls can then present their tumor antigens to the patient's immune system, but cannot multiply to create new tumors.

Alternatively, tumor cells that can be cultured may be transduced with a suicide gene. As described above, a gene such as the herpes simplex thymidine kinase (TK) gene can be transferred into tumor cells to induce their destruction by administration of acyclovir or gancyclovir. After immunization, the TK expressing tumor cells can present their tumor antigens, and are capable of proliferation. After a period of time during which the patients's immune response is stimulated, the cells can be selectively killed. This approach might allow longer viability of the tumor cells utilized for immunizations, which may be advantageous in the induction or augmentation of anti-tumor immunity.

(F) Preparation of Samples for Immunization

CE cells and/or carrier cells and tumor cells, and/or homogenates of tumor cells and/or purified tumor antigen(s), are combined for patient immunization. Approximately  $10^7$  tumor cells will be required. If homogenates of tumor cells or purified or non-purified fractions of tumor antigens are used, the tumor dose can be adjusted based on the normal number of tumor antigens usually present on  $10^7$  intact tumor cells. The tumor preparation should be mixed with numbers of CE or carrier

cells sufficient to secrete cytokin levels that induce anti-tumor immunity (11-12) without producing substantial systemic toxicity which would interfere with therapy.

5 The cytokines should be produced by the CE cells or the carrier cells at levels sufficient to induce or augment immune response but low enough to avoid substantial systemic toxicity. This prevents side effects created by previous methods' administration of greater than physiological levels of the cytokines.

10 These mixtures, as well as carrier cells that are utilized alone, will be formulated for injection in any manner known in the art acceptable for immunization. Because it is important that at least the CE cells and carrier cells remain viable, the formulations must be  
15 compatible with cell survival. Formulations can be injected subcutaneously, intramuscularly, or in any manner acceptable for immunization.

Contaminants in the preparation which may focus the immune response on undesired antigens should be removed  
20 prior to the immunizations.

The following examples are provided for illustration of several embodiments of the invention and should not be interpreted as limiting the scope of the invention.

EXAMPLE IIMMUNIZATION WITH FIBROBLASTS EXPRESSING IL-2  
MIXED WITH IRRADIATED TUMOR CELLS5      1) Isolation of Autologous Fibroblasts  
for Use in Generating IL-2 Secreting CE Cells

Skin punch biopsies will be obtained from each patient under sterile conditions. The biopsy tissue will be minced and placed in RPMI 1640 media containing 10% fetal calf serum (or similar media) to establish growth of the skin fibroblasts in culture. The cultured fibroblasts will be utilized to generate IL-2 secreting CE cells by retroviral mediated IL-2 gene transfer.

2) Retroviral Vector Preparation and  
Generation of IL-2 Secreting CE Cells

15      The cultured skin fibroblasts will then be infected with a retroviral vector containing the IL-2 and Neomycin resistance (Neo<sup>R</sup>) genes. An N2 vector containing the Neo<sup>R</sup> gene will be used, and has been previously utilized by a number of investigators for in vitro and in vivo work, including investigations with human subjects (16). The IL-2 vector will be generated from an N2-derived vector, LLRNL, developed and described by Friedmann and his colleagues (20). It will be made by replacement of the luciferase gene of LLRNL with a full-length cDNA encoding human IL-2. Retroviral vector free of contaminating replication-competent virus is produced by transfection of vector plasmid constructions into the helper-free packaging cell line PA317. Before infection of patients' cells, the vector will have been shown to be free of helper virus. In the event that helper virus is detected, the vector will be produced in the GP + envAM12 packaging cell line in which

the viral gag and pol genes are separated from the env, further reducing the likelihood of helper virus production.

### 3) Transduction Protocol

The cultured primary fibroblasts will be incubated with supernatant from the packaging cell line as described (20). Supernatant from these cells will be tested for adventitious agents and replication competent virus as described (16) and outlined in Table 1. The fibroblasts are washed and then grown in culture media containing G418, (a neomycin analogue) to select for transduced cells expressing the Neo<sup>R</sup> gene. The G418-resistant cells will be tested for expression of the IL-2 gene by measuring the concentration of IL-2 in the culture supernatant by an enzyme linked immunosorbent assay (ELISA) (12). G418-resilient cells expressing IL-2 will be stored at -70°C until required for subsequent use in immunizations.

Table 1

#### Adventitious Agents and Safety Testing

20	1. Sterility
	2. Mycoplasma
	3. General Safety
	4. Viral Testing
	LCM Virus
25	Thymic agent
	S+/L- eco
	S+/L-xeno
	S+/L- amphi
	3T3 amplification
30	MRC-5/Vero

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#### 4) Preparation of Irradiated Tumor Cells

Tumors obtained from clinically indicated surgical resections or from superficial lymph node or skin metastases will be minced into 2-3 mm pieces and treated with collagenase and DNase to facilitate separation of the tumor into a single cell suspension. The collected cells will be centrifuged and washed in RPMI 1640 media and then cryopreserved in a solution containing 10% dimethyl sulphoxide and 50% fetal calf serum in RPMI 1640 media. The cells will be stored in liquid nitrogen until the time of administration. Prior to their use in subcutaneous immunizations, the cells will be thawed, washed in media free of immunogenic contaminants, and irradiated with 4,000 rads per minute for a total of 20,000 rads in a cesium irradiator.

#### 5) Patient Selection

Patients will have a histologically confirmed diagnosis of cancer. Patients with tumors that must be resected for therapeutic purposes or with tumors readily accessible for biopsy are most appropriate for this embodiment of the invention.

#### 6) Pretreatment Evaluation

The following pretreatment evaluations will be performed:

- 1) History and physical examination including a description and quantification of disease activity.

## 2) Performance Status Assessment

- 0 = Normal, no symptoms  
1 = Restricted, but ambulatory  
2 = Up greater than 50% of waking hours, capable of self-care  
3 = Greater than 50% of waking hours confined to bed or chair, limited self-care  
4 = Bedridden

5

## 3) Pretreatment Laboratory:

CBC with differential, platelet count, PT, PTT, glucose, BUN, creatinine, electrolytes, SGOT, SGPT, LDH, alkaline phosphatase, bilirubin, uric acid, calcium, total protein albumin.

10

## 4) Other Analyses:

Urinalysis

CH<sub>50</sub>, C<sub>3</sub> and C<sub>4</sub> serum complement levels

Immunophenotyping of peripheral blood B cell and T cell subsets

15

Assays for detectable replication-competent virus in peripheral blood cells

PCR assays of peripheral blood leukocytes for Neo<sup>R</sup>, IL-2 and viral env

## 5) Other Pretreatment Evaluation:

20

Chest X-ray and other diagnostic studies including computerized tomography (CT), magnetic resonance imaging (MRI) or radionuclide scans may be performed to document and quantify the extent of disease activity.

25

Follow-up evaluations of these assessments at regular intervals during the course of therapy (approximately every 1 to 3 months) will be useful in determining response to therapy and potential toxicity,

permitting adjustments in the number of immunizations administered.

#### 7) Restrictions on Concurrent Therapy

For optimal effects of this treatment, patients  
5 should receive no concurrent therapy which is known to suppress the immune system.

#### 8) Final Formulation

Each patient will receive subcutaneous immunizations with a mixture of irradiated tumor cells and  
10 autologous fibroblast CE cells genetically modified to secrete IL-2. Approximately  $10^7$  tumor cells will be mixed with  $10^7$  fibroblasts known to secrete at least 20 units/ml of IL-2 in tissue culture when semi-confluent (12). The irradiated tumor cells and genetically modified fibroblasts  
15 will be placed in a final volume of 0.2 ml normal saline for immunization.

#### 9) Dose Adjustments

At least two subcutaneous immunizations will be administered, two weeks apart, with irradiated tumor cells  
20 and autologous fibroblasts genetically modified to secrete IL-2. If no toxicity is observed, subsequent booster immunizations may be administered periodically (at least one week apart) to optimize the anti-tumor immune response.

#### J) Treatment of Potential Toxicity

25 Toxic side effects are not expected to result from these immunizations. However, potential side effects of these immunizations are treatable in the following manner:

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If massive tumor cell lysis results, any resulting uric acid nephropathy, adult respiratory distress syndrome, disseminated intravascular coagulation or hyperkalemia will be treated using standard methods.

- 5           Local toxicity at the sites of immunization will be treated with either topical steroids and/or surgical excision of the injection site as deemed appropriate.

- Hypersensitivity reactions such as chills, fever and/or rash will be treated symptomatically with  
10 antipyretics and antihistamines. Patients should not be treated prophylactically. Should arthralgias, lymphadenopathy or renal dysfunction occur, treatment with corticosteroids and/or antihistamines will be instituted. Anaphylaxis will be treated by standard means such as  
15 administration of epinephrine, fluids, and steroids.

#### EXAMPLE II

A.           Retroviral IL-2 Gene Transfer and Expression in Fibroblasts

- Retroviral vectors were employed to transfer and  
20 express IL-2 and neomycin phosphotransferase genes in murine and primary human fibroblasts. The retroviral vector DC/TKIL2 produced by Gilboa and co-workers (Gansbacher, et al., J. Exp. Med. 172:1217-1223, 1990, which is incorporated herein by reference) was utilized to  
25 transduce murine fibroblasts for application in an animal tumor model (see Section B below). Human fibroblasts were transduced with the retroviral vector LXSN-RI-IL2. Schematic diagrams of the structure of these retroviral vectors are provided in Figure 1. A more complete  
30 description of the LXSN-RI-IL2 vector, including its nucleotide sequence, is provided in Example III and in Tables 2, 3 and 4.

Following infection with the described vectors and selection for 2-3 weeks in growth media containing the neomycin analogue G418, Balb/c and human embryonic fibroblast culture supernatants were harvested and tested for IL-2 by an enzyme-linked immunosorbent assay (ELISA). Figure 2 depicts the levels of IL-2 secreted by the transduced fibroblasts.

These results can be confirmed using negative control fibroblasts infected with an N2-derived retroviral vector expressing an irrelevant gene such as luciferase or  $\beta$ -galactosidase and studies with adult human fibroblasts.

Biological activity of the IL-2 expressed by the transduced human fibroblasts was confirmed by a cell proliferation bioassay employing an IL-2 dependent T cell line. In this assay, supernatant from the transduced fibroblasts and control unmodified fibroblasts were incubated with the IL-2 dependent T cell line CTLL-2. Incorporation of  $^3\text{H}$ -thymidine was measured as an indicator of cell proliferation and IL-2 activity (Figure 3).

B. Efficacy of Transduced Fibroblasts in an Animal Tumor Model

The efficacy of fibroblasts genetically modified to secrete IL-2 was tested in an animal model of colorectal carcinoma. In these studies, the Balb/c CT26 tumor cell line was injected subcutaneously with Balb/c fibroblasts transduced to express IL-2. Control groups included animals injected with 1) a mixture of CT26 tumor cells and unmodified fibroblasts; 2) CT26 tumor cells without fibroblasts and 3) transduced fibroblasts alone. No tumors were detected in 3/8 animals treated with transduced fibroblasts and CT26 cells. In contrast, all untreated control animals (8/8) injected with CT26 tumor cells developed palpable tumors. No tumors were detected in the

animals inoculated with transduced fibroblasts without CT26 tumor cells. The mean CT26 tumor size in Balb/c mice injected with the IL-2 secreting fibroblasts was considerably smaller compared to the control groups (Figure 4). A multivariate non-parametric statistical procedure (Koziol, et al., Biometrics 37:383-390, 1981 and Koziol, et al., Computer Prog. Biomed. 19:69-74, 1984, which is incorporated herein by reference) was utilized to evaluate differences in tumor growth among the treatment groups. The tumor growth curves for the four treatment groups presented in Figure 4 were significantly different ( $p=0.048$ ). Subsequent comparisons between treatment groups revealed a significant difference ( $p < 0.05$ ) in tumor growth between animals injected with CT26 tumor cells alone and animals treated with  $2 \times 10^6$  transduced fibroblasts and CT26 tumor cells (Figure 4).

### EXAMPLE III

#### A. Project Overview

Lymphokine gene therapy of cancer will be evaluated in cancer patients who have failed conventional therapy. An N2-derived vector containing the neomycin phosphotransferase gene will be used. This vector has been employed by a number of investigators for in vitro and in vivo studies including recently approved investigations with human subjects (Rosenberg et al., N. Eng. J. Med., 323:570-578, 1990). The lymphokine vectors used in this investigation will be generated from the N2-derived vector, LXS<sub>N</sub>, developed and described by Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989, which are incorporated herein by reference. The vector LXS<sub>N</sub>-RI-IL2 contains human IL-2 cDNA under the control of the retroviral 5' LTR promoter and the neomycin phosphotransferase gene under the control of the SV40 promoter (see Figure 1). The normal human IL-2 leader

sequence has been replaced with a chimeric sequence containing rat insulin and human IL-2 leader sequences (see Tables 2, 3 and 4). This chimeric leader sequence enhances IL-2 gene expression.

5           To construct the LXSN-RI-IL2 vector, the bacterial plasmid pBC12/CMV/IL2 (Cullen, B.R., DNA 7:645-650, 1988, which is incorporated herein by reference) containing the full-length IL-2 cDNA and chimeric leader  
10           sequence was digested with HindIII and the ends were blunted using Klenow polymerase. IL-2 cDNA was subsequently released from the plasmid by digestion with BamHI. The IL-2 fragment was purified by electrophoresis in a 1% agarose gel and the appropriate band was extracted  
15           utilizing a glass powder method. Briefly, the gel slice was dissolved in 4M NaI at 55°. After cooling to room temperature, 4  $\mu$ l of oxidized silica solution (BIO-101, La Jolla, CA) was added to adsorb the DNA. The silica was  
20           ythen washed with a cold solution of 50% ethanol containing 0.1 M NaCl in TE buffer. The DNA was eluted from the silica by heating at 55° in distilled H<sub>2</sub>O. The purified IL-2 cDNA was then directionally ligated into the HpaI-BamHI cloning sites of the pLXSN vector. A more complete description of the pLXSN-RI-IL2 vector and its partial nucleotide sequence are provided in Tables 2, 3, 4, 5 and  
25           6.

Table 2**Description of the LXSNI-RI-IL2  
from position 1 to 6365**

<u>Bases</u>	<u>Description</u>
1-589	Moloney murine sarcoma virus 5' LTR
659-1458	The sequence of the extended packaging signal
1469-2151	IL-2 cDNA with chimeric leader sequence
1469-1718	IL-2 chimeric leader sequence
1647-1718	coding region of the signal peptide
1719-2151	Mature IL-2 coding sequence
2158-2159	Mo mu sarcoma virus end/SV 40 start
2159-2503	Simian virus 40 early promoter
2521-2522	Simian virus DNA end/Tn5 DNA start
2557-3351	Neomycin phosphotransferase
3370-3371	Tn5 DNA end/Moloney murine leukemia virus start
3411-4004	Moloney murine leukemia virus 3' LTR
4073-4074	Moloney murine leukemia DNA end/pBR322 DNA start
4074-6365	Plasmid backbone

Table 3

Enzyme [# Cuts]		Position(s)	
Aat1	[ 2]	1961,	2481
Aat2	[ 2]	811,	6295
Acc1	[ 1]	4252	
Acc2	[ 19]	392,	394,
		445,	969,
		971,	1193,
		2751,	3052,
		3084,	3807,
		3809,	4081,
		4083,	
		4186,	4527,
		5108,	5438,
		5931,	6263
Acy1	[ 5]	808,	2685,
		3860,	5910,
		6292	
Afl1	[ 13]	260,	273,
		328,	626,
		756,	1277,
		3201,	3676,
		3689,	3744,
		4041,	5511,
		5733	
Afl2	[ 4]	34,	1064,
		1955,	3446
Afl3	[ 2]	1592,	4480
Aha1	[ 20]	161,	237,
		473,	474,
		602,	644,
		789,	2689,
		2849,	3578,
		3653,	3888,
		3889,	
		4017,	4059,
		4126,	4161,
		4860,	5556,
		5907	
Aha2	[ 5]	808,	2685,
		3860,	5910,
		6292	
Aha3	[ 3]	5239,	5258,
		5950	
Alu1	[ 33]	29,	33,
		119,	190,
		411,	654,
		734,	742,
		1470,	1486,
		1751,	1935,
		2003,	2446,
		2500,	2791,
		3249,	3441,
		3445,	3532,
		3607,	
		3826,	4069,
		4122,	4141,
		4422,	4648,
		4738,	
		4784,	5041,
		5562,	5662,
		5725	
Alw1	[ 20]	1110,	1414,
		1665,	2018,
		2147,	2160,
		2529,	2553,
		2864,	2929,
		3110,	4027,
		5041,	
		5127,	5129,
		5225,	5226,
		5689,	6006,
		6010	
AlwN1	[ 4]	231,	3572,
		3647,	4896
Aoc1	[ 2]	847,	1076
Aoc2	[ 19]	323,	413,
		426,	597,
		1583,	1721,
		2631,	2724,
		2798,	2988,
		3050,	3739,
		3828,	
		3841,	4012,
		4300,	4798,
		5959,	6044
Aos1	[ 2]	2787,	5595
ApaL1	[ 4]	1717,	4296,
		4794,	6040

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Apy1	[ 22]	315,	623,	801,	814,	1227,	1252,
		1275,	1295,	1325,	1526,	1536,	1558,
		2196,	2251,	2268,	3072,	3731,	4038,
		4629,	4642				4508,
Aqu1	[ 6]	241,	472,	1998,	3821,	3854,	3887
Asel	[ 2]	1801,	5545				
Asp700	[ 1]	5972					
Asp718	[ 2]	476,	3891				
AspA1	[ 1]	1145					
Asu1	[ 29]	169,	200,	245,	260,	273,	328,
		626,	756,	826,	839,	1043,	1254,
		1532,	1649,	3201,	3541,	3586,	3616,
		3676,	3689,	3744,	4041,	5415,	5494,
		5733,	6349				5511,
Ava1	[ 6]	241,	472,	1998,	3821,	3854,	3887
Ava2	[ 13]	260,	273,	328,	626,	756,	1277,
		3201,	3676,	3689,	3744,	4041,	5511,
							5733
Ava3	[ 2]	2232,	2304				
Avr2	[ 2]	1962,	2482				
Ba11	[ 3]	658,	1169,	2767			
BamH1	[ 1]	2152					
Ban1	[ 9]	318,	476,	1200,	2684,	2719,	3734,
		3859,	3891,	5321			
Ban2	[ 8]	413,	426,	597,	1583,	3050,	3828,
		3841,	4012				
Bbe1	[ 2]	2688,	3863				
Bbv1	[ 22]	969,	997,	1738,	2493,	2632,	2758,
		2800,	2816,	2909,	3321,	4060,	4131,
		4372,	4390,	4809,	4899,	4902,	5108,
		5600,	5802				5411,
Bcl1	[ 1]	2526					
Bgl1	[ 2]	2435,	5493				
Bspl286I	[ 19]	323,	413,	426,	597,	1583,	1721,
		2631,	2724,	2798,	2988,	3050,	3739,
		3841,	4012,	4300,	4798,	5959,	6044

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BspH1	[	3]	5200,	6208,	6313			
BspM1	[	4]	1501,	2500,	2572,	2953		
BssH2	[	4]	392,	443,	3082,	3807		
BstE2	[	1]	1145					
BstN1	[	22]	315,	623,	801,	814,	1227,	1252,
			1275,	1295,	1325,	1526,	1536,	1558,
			2196,	2251,	2268,	3072,	3731,	4038,
			4629,	4642				
BstU1	[	19]	392,	394,	445,	969,	971,	1193,
			2751,	3052,	3084,	3807,	3809,	4081,
			4186,	4527,	5108,	5438,	5931,	6263
BstX1	[	1]	2060					
BstY1	[	11]	2010,	2152,	2521,	2856,	3102,	5121,
			5132,	5218,	5230,	5998,	6015	
Bsu36I	[	2]	847,	1076				
Ccr1	[	1]	1998					
Cfo1	[	31]	394,	396,	445,	447,	714,	971,
			2679,	2687,	2751,	2788,	3054,	3084,
			3314,	3809,	3811,	3862,	4083,	4186,
			4357,	4390,	4660,	4727,	4827,	5001,
			5503,	5596,	5933,	6265		
Cfr1	[	9]	656,	790,	1167,	1188,	2591,	2765,
			3156,	3183,	5761			
Cfr10I	[	3]	3004,	3185,	5453			
Cfr13I	[	29]	169,	200,	245,	260,	273,	328,
			626,	756,	826,	839,	1043,	1254,
			1532,	1649,	3201,	3541,	3586,	3616,
			3676,	3689,	3744,	4041,	5415,	5494,
			5733,	6349				
Cvn1	[	2]	847,	1076				
Dde1	[	23]	75,	165,	191,	282,	553,	847,
			1076,	1348,	1692,	2442,	3348,	3487,
			3657,	3698,	3879,	3967,	4290,	4755,
			5330,	5870,	6296			
Dpn1	[	30]	95,	1104,	1236,	1421,	1659,	2012,
			2154,	2523,	2528,	2547,	2858,	2936,
			3026,	3104,	3507,	4021,	5048,	5123,
			5142,	5220,	5232,	5337,	5678,	5696,
			6000,	6017,	6053			

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Dra1	[ 3]	5239,	5258,	5950			
Dra2	[ 4]	328,	1277,	3744,	6349		
Eae1	[ 9]	656,	790,	1167,	1188,	2591,	2765,
		3156,	3183,	5761			
Eag1	[ 2]	790,	2591				
Eco47I	[ 13]	260,	273,	328,	626,	756,	1277,
		3201,	3676,	3689,	3744,	4041,	5511,
					5733		
Eco52I	[ 2]	790,	2591				
Eco81I	[ 2]	847,	1076				
EcoN1	[ 2]	850,	1450				
EcoO109I	[ 4]	328,	1277,	3744,	6349		
EcoR1	[ 1]	1460					
EcoR1*	[ 14]	938,	1037,	1460,	1798,	1805,	1928,
		2064,	2121,	2236,	2308,	2400,	5240,
		5801				5546,	
EcoR2	[ 22]	313,	621,	799,	812,	1225,	1250,
		1273,	1293,	1323,	1524,	1534,	1556,
		2194,	2249,	2266,	3070,	3729,	4036,
		4627,	4640			4506,	
EcoR5	[ 4]	137,	213,	3554,	3629		
EcoT22I	[ 2]	2232,	2304				
Fdi2	[ 2]	2787,	5595				
Fnu4H1	[ 41]	793,	967,	983,	986,	1191,	1752,
		2430,	2507,	2594,	2646,	2657,	2747,
		2789,	2830,	2917,	2920,	2923,	3159,
		3296,	3310,	4074,	4120,	4217,	4270,
		4404,	4407,	4525,	4680,	4823,	4888,
		5097,	5425,	5614,	5764,	5791,	5886,
						6115	
FnuD2	[ 19]	392,	394,	445,	969,	971,	1193,
		2751,	3052,	3084,	3807,	3809,	4081,
		4186,	4527,	5108,	5438,	5931,	6263
Fok1	[ 13]	498,	1198,	1358,	1679,	2333,	2552,
		3009,	3034,	3912,	4168,	5339,	5520,
						5807	
Fsp1	[ 2]	2787,	5595				
Hae2	[ 4]	2688,	3863,	4358,	4728		

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Ha 3	[ 35]	171,	202,	247,	658,	792,	828,
	840,	1045,	1169,	1190,	1255,	1534,	1650,
	1866,	1961,	2423,	2429,	2438,	2481,	2593,
	2767,	3158,	3185,	3543,	3588,	3618,	3663,
	4495,	4506,	4524,	4958,	5416,	5496,	5763,
	6350						
Hap2	[ 30]	161,	237,	473,	601,	643,	789,
	2590,	2667,	2689,	2717,	2848,	2938,	3005,
	3186,	3578,	3653,	3888,	4016,	4058,	4126,
	4160,	4687,	4834,	4860,	5050,	5454,	5488,
	5555,	5665,	5907				
Hga1	[ 8]	455,	707,	960,	1580,	4175,	4591,
	5169,	5899					
HgiA1	[ 9]	413,	1721,	2798,	2988,	3828,	4300,
	4798,	5959,	6044				
Hha1	[ 31]	394,	396,	445,	447,	714,	971,
	2679,	2687,	2751,	2788,	3054,	3084,	3086,
	3314,	3809,	3811,	3862,	4083,	4186,	4216,
	4357,	4390,	4660,	4727,	4827,	5001,	5110,
	5503,	5596,	5933,	6265			
HinP1	[ 31]	392,	394,	443,	445,	712,	969,
	2677,	2685,	2749,	2786,	3052,	3082,	3084,
	3312,	3807,	3809,	3860,	4081,	4184,	4214,
	4355,	4388,	4658,	4725,	4825,	4999,	5108,
	5501,	5594,	5931,	6263			
Hinc2	[ 1]	5914					
Hind2	[ 1]	5914					
Hind3	[ 1]	2498					
Hinf1	[ 14]	298,	517,	857,	868,	1553,	1814,
	3170,	3304,	3356,	3881,	4380,	4455,	4851,
	5368						
Hpa2	[ 30]	161,	237,	473,	601,	643,	789,
	2590,	2667,	2689,	2717,	2848,	2938,	3005,
	3186,	3578,	3653,	3888,	4016,	4058,	4126,
	4160,	4687,	4834,	4860,	5050,	5454,	5488,
	5555,	5665,	5907				
Hph1	[ 11]	1214,	1240,	1817,	2863,	4102,	4111,
	5216,	5443,	5859,	6065,	6100		
Kpn1	[ 2]	480,	3895				
Mae1	[ 15]	30,	293,	689,	727,	739,	1452,
	1606,	1893,	1963,	2483,	3442,	3709,	4975,
	5228,	5563					

Mae2	[ 11]	808,	1139,	1180,	1987,	2801,	2988,
		4233,	5183,	5599,	5972,	6292	
Mae3	[ 20]	38,	1052,	1080,	1145,	1289,	1478,
		1706,	2805,	3111,	3450,	4134,	4229,
		4899,	5015,	5298,	5629,	5687,	5840,
							6028
Mbo1	[ 30]	93,	1102,	1234,	1419,	1657,	2010,
		2152,	2521,	2526,	2545,	2856,	2934,
		3024,	3102,	3505,	4019,	5046,	5121,
		5140,	5218,	5230,	5335,	5676,	5694,
		5998,	6015,	6051			
Mbo2	[ 17]	444,	1145,	1356,	1575,	1617,	1908,
		1911,	3046,	3256,	3336,	4351,	5142,
		5968,	6046,	6155,	6351		
Mnl1	[ 54]	291,	444,	508,	534,	560,	639,
		841,	939,	1227,	1330,	1363,	1369,
		1378,	1408,	1411,	1426,	1433,	1449,
		1620,	1909,	1921,	2412,	2418,	2443,
		2455,	2458,	2470,	2508,	2535,	2599,
		3092,	3286,	3707,	3859,	3878,	3923,
		3974,	4054,	4087,	4117,	4379,	4587,
		4911,	5311,	5392,	5540,	5746,	6339
Mse1	[ 22]	35,	1065,	1177,	1207,	1231,	1801,
		1843,	1956,	1971,	2124,	2139,	3447,
		5186,	5238,	5243,	5257,	5310,	5545,
		5949,	6321				
Msp1	[ 30]	161,	237,	473,	601,	643,	789,
		2590,	2667,	2689,	2717,	2848,	2938,
		3186,	3578,	3653,	3888,	4016,	4058,
		4160,	4687,	4834,	4860,	5050,	5454,
		5555,	5665,	5907			
Mst1	[ 2]	2787,	5595				
Mst2	[ 2]	847,	1076				
Mval	[ 22]	315,	623,	801,	814,	1227,	1252,
		1275,	1295,	1325,	1526,	1536,	1558,
		2196,	2251,	2268,	3072,	3731,	4038,
		4629,	4642				
Nae1	[ 1]	3187					
Nar1	[ 2]	2685,	3860				
Nci1	[ 20]	161,	237,	473,	474,	602,	644,
		789,	2689,	2849,	3578,	3653,	3888,
		4017,	4059,	4126,	4161,	4860,	5556,
							5907
Nco1	[ 2]	2389,	3117				

Nde1	[	1]	4303						
Nde2	[	30]	93,	1102,	1234,	1419,	1657,	2010,	
			2152,	2521,	2526,	2545,	2856,	2934,	3015,
			3024,	3102,	3505,	4019,	5046,	5121,	5132,
			5140,	5218,	5230,	5335,	5676,	5694,	5740,
			5998,	6015,	6051				
Nhe1	[	3]	29,	1605,	3441				
Nla3	[	26]	61,	1263,	1596,	1649,	1835,	1856,	
			2030,	2230,	2302,	2393,	2559,	2904,	3090,
			3121,	3147,	3473,	4119,	4224,	4484,	5204,
			5695,	5705,	5783,	5819,	6212,	6317	
Nla4	[	28]	153,	246,	262,	320,	478,	627,	
			758,	827,	959,	1202,	1279,	2154,	2200,
			2272,	2686,	2721,	3678,	3736,	3861,	3893,
			4042,	4512,	4551,	5323,	5417,	5458,	5669,
			6259						
Nsi1	[	2]	2232,	2304					
Nsp(7524)1	[	8]	1596,	1835,	1856,	2230,	2302,	3090,	
			4119,	4484					
Nsp(7524)2	[	19]	323,	413,	426,	597,	1583,	1721,	
			2631,	2724,	2798,	2988,	3050,	3739,	3828,
			3841,	4012,	4300,	4798,	5959,	6044	
NspB2	[	12]	119,	190,	1751,	2158,	2791,	3532,	
			3607,	3989,	4192,	4822,	5067,	6008	
NspH1	[	8]	1596,	1835,	1856,	2230,	2302,	3090,	
			4119,	4484					
Paer7I	[	1]	1998						
Pall	[	35]	171,	202,	247,	658,	792,	828,	
			840,	1045,	1169,	1190,	1255,	1534,	1650,
			1866,	1961,	2423,	2429,	2438,	2481,	2593,
			2767,	3158,	3185,	3543,	3588,	3618,	3663,
			4495,	4506,	4524,	4958,	5416,	5496,	5763,
			6350						
Ple1	[	7]	865,	1547,	3350,	3889,	4374,	4859,	
			5362						
PpuM1	[	3]	328,	1277,	3744				
Pss1	[	4]	331,	1280,	3747,	6352			
Pst1	[	6]	987,	1163,	1888,	2511,	2738,	5618	
Pvu1	[	1]	5743						

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Pvu2	[ 6]	119,	190,	1751,	2791,	3532,	3607
Rsa1	[ 10]	347,	478,	725,	1342,	1519,	1597,
	2991,	3893,	4288,	5853			
Rsr2	[ 1]	3201					
Sac1	[ 2]	413,	3828				
Sau1	[ 2]	847,	1076				
Sau3A1	[ 30]	93,	1102,	1234,	1419,	1657,	2010,
	2152,	2521,	2526,	2545,	2856,	2934,	3015,
	3024,	3102,	3505,	4019,	5046,	5121,	5132,
	5140,	5218,	5230,	5335,	5676,	5694,	5740,
	5998,	6015,	6051				
Sau96I	[ 29]	169,	200,	245,	260,	273,	328,
	626,	756,	826,	839,	1043,	1254,	1277,
	1532,	1649,	3201,	3541,	3586,	3616,	3661,
	3676,	3689,	3744,	4041,	5415,	5494,	5511,
	5733,	6349					
Scal	[ 1]	5853					
ScrF1	[ 42]	161,	237,	315,	473,	474,	602,
	623,	644,	789,	801,	814,	1227,	1252,
	1295,	1325,	1526,	1536,	1558,	1630,	2196,
	2251,	2268,	2689,	2849,	3072,	3578,	3653,
	3731,	3888,	3889,	4017,	4038,	4059,	4126,
	4161,	4508,	4629,	4642,	4860,	5556,	5907
Sdu1	[ 19]	323,	413,	426,	597,	1583,	1721,
	2631,	2724,	2798,	2988,	3050,	3739,	3828,
	3841,	4012,	4300,	4798,	5959,	6044	
Sec1	[ 38]	159,	235,	314,	324,	472,	536,
	621,	622,	760,	799,	800,	812,	813,
	1294,	1303,	1323,	1324,	1525,	1557,	1962,
	2194,	2266,	2389,	2424,	2433,	2482,	2848,
	3117,	3576,	3651,	3730,	3740,	3887,	3950,
	4036,	4037,	4640				
SfaN1	[ 23]	258,	520,	997,	1657,	2107,	2239,
	2311,	2643,	2898,	2984,	3048,	3114,	3323,
	3674,	3934,	4146,	4281,	4317,	4357,	4577,
	5629,	5820,	6069				
Sfi1	[ 1]	2435					
Sma1	[ 2]	474,	3889				
Spe1	[ 1]	726					
Sph1	[ 4]	1835,	2230,	2302,	3090		

Ssp1	[	1]	6177						
Sst1	[	2]	413,	3828					
Stu1	[	2]	1961,	2481					
Sty1	[	9]	324,	536,	1303,	1962,	2389,	2482,	
			3117,	3740,	3950				
Taq1	[	15]	860,	1096,	1407,	1418,	1660,	1999,	
			2514,	2798,	2954,	2978,	3014,	3176,	3367,
			4580,	6024					
Tha1	[	19]	392,	394,	445,	969,	971,	1193,	
			2751,	3052,	3084,	3807,	3809,	4081,	4083,
			4186,	4527,	5108,	5438,	5931,	6263	
Tth111I	[	6]	465,	877,	1275,	2803,	3880,	4227	
Xba1	[	2]	1892,	3708					
Xho1	[	1]	1998						
Xho2	[	11]	2010,	2152,	2521,	2856,	3102,	5121,	
			5132,	5218,	5230,	5998,	6015		
Xma1	[	2]	472,	3887					
Xma3	[	2]	790,	2591					
Xmn1	[	1]	5972						
Xor2	[	1]	5743						

Table 4

Enzymes which do not cut LXS NR II.L2:

Acc3	Bgl2	Cla1	Hpa1	Nru1
SnaB1				
Apa1	Bsm1	Dra3	Mlu1	PflM1
Sp11				
Asu2	BspM2	Eco47III	Mro1	Sac2
Sst2				
Ban3	BstB1	Esp1	Not1	Sal1



Asu1	--33--	112--	1-11--	11--	+	1--	241--	1--	12--	1--	1--	1--
Ava1	--1--	1--	1--	1--	+	1--	21--	1--	1--	1--	1--	1--
Ava2	--3--	11--	1--	1--	+	1--	21--	1--	1--	1--	1--	1--
Ava3	--	1--	1--	11--	+	1--	1--	1--	1--	1--	1--	1--
Avr2	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Ball	--1--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
BamH1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Ban1	--1-1--	1--	1--	11--	+	111--	1--	1--	1--	1--	1--	1--
Ban2	--11-1--	1--	1--	1--	+	2-1--	1--	1--	1--	1--	1--	1--
Bbe1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Bbv1	--2--	1--	1--	1-1121--	+	1--	111-2--	12+1--	1-1--	1--	1--	1--
Bcl1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Bgl1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Bsp1286I	--111-1--	1--	1--	111-11--	+	12-1--	1--	1--	1--	1--	1--	1--
BspH1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
BspM1	--11--	1--	1--	11--	+	1--	1--	1--	1--	1--	1--	1--
BssH2	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
BstE2	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
BstN1	--1-1-2--	23--	31--	3--	+	1--	1--	1-2--	1--	1--	1--	1--
BstU1	--21--	2--	1--	1--	+	1-2--	2+21--	1--	1--	1--	1--	1--
BstX1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
BstY1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Bsu36I	--1+1--	1--	1--	1--	+	1--	1--	1--	112--	2--	1--	1--
Ccr1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Cfo1	--22--	1--	1--	211+3--	+	1--	3+11111--	111-11--	11--	1--	1--	1--
Cfr1	--1-1--	1--	1--	1--	+	1-1--	2--	1--	1--	1--	1--	1--
Cfr10I	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Cfr13I	--33--	112--	1-11--	11--	+	1--	241--	1--	12--	1--	1--	1--
Cvnl	--	1+1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Ddel	1111--	1--	1--	1--	+	1-1111-11--	1--	1--	1-1--	1--	1--	1--
Dpn1	--1--	1--	1--	1--	+	3--	212--	1--	2221--	21--	21--	1--
Dra1	--	1--	1--	1--	+	1--	1--	1--	2--	1--	1--	1--
Dra2	--1--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Eae1	--1-1--	1--	1--	1--	+	1-1--	2--	1--	1--	1--	1--	1--
Eag1	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--
Eco47I	--3--	11--	1--	1--	+	1--	21--	1--	1--	1--	1--	1--
Eco52I	--	1--	1--	1--	+	1--	1--	1--	1--	1--	1--	1--

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[illegible]

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Table 5 (Cont'd)



from 1 to 6365. Numbered from position 1.

```

                                >NheI   >AflII
                                |       |
mouse_DNA_end/HO-MuSV_DNA_start_[split]
|       .   10       .   20       .   30       .   40       .   50       .   60       .   70
v       v       v       v       v       v       v       v
TTTGAAGAC CCCACCGTA GGTGCCAAGC TAGCTTAAGT AACGCCACTT TGCAGGCCAT CGAAAAATAC
AACTTTCTG GGTGCGCAT CCACCGTTC ATCGAATTCA TTCCGGTGAA AGCTTCOGTA CCTTTTATG

                                >PvuII
                                |
                                >NspB2
                                |
                                >EcoRI
                                |
                                .   80       .   90       .   100      .   110      .   120      .   130      .   140
                                v       v       v       v       v       v       v
ATAACTGACA ATAGGAAAGT TCAGATCAAG GTCAGGAACA AACAAACAGC TGAATACCA ACAGGATATC
TATTGACTCT TATCGTTTCA AGTCTAGTTC CAGTCTTGT TTCTTTGTGC ACTTATGGTT TGTCTATAG

                                >PvuII
                                |
                                >NspB2
                                |
                                .   150      .   160      .   170      .   180      .   190      .   200      .   210
                                v       v       v       v       v       v       v
TGTGGTAAGC GGTTCCTGCC CCGGCTCAGG CCCAAGAACA CATGACACAG CTCAGTGATG GGGCAACAG
ACACCATTCG CCAAGCAAGG GCGCGAGTCC CGGTTCTTGT CTACTCTGTC GACTCACTAC CCGGTTTGTG

                                >AvaI
                                |
                                >EcoRI
                                |
                                >AluNI
                                |
                                >AguI
                                |
                                .   220      .   230      .   240      .   250      .   260      .   270      .   280
                                v       v       v       v       v       v       v
GATATCTCTG GTAGCAGTT CCGGCCCCGG CTGGGGGCGA AGAACAGATG GTGCCAGAT GCGGTCCAGC
CTATAGACAC CATTCGTCAA CCAACGGGCG GAGCCCGCGT TCTTGCTTAC CAGCGGTCTA CCGCAGGTG

                                >PpuMI
                                |
                                >DraI
                                |
                                >EcoO109I
                                |
                                >BamI >SylI >PssI >RsaI
                                |   |   |   |
                                .   290      .   300      .   310      .   320      .   330      .   340      .   350
                                v       v       v       v       v       v       v
CCTCAGCAGT TTCTAGTCAA TCATCAGATG TTTCAGCGT GCGGCCAAGA CCTGAAAATG ACCCTGTACG
GCACTGCTCA AAGATCACTT AGTAGTCTAC AAAAGTCCCA CCGCGTTCTT GCACTTTTAC TCGACATCG

```

```

      >Ban2
      .
      >Sac1
      .
      >Sst1
      .
      >HgiA1
      .
      >BssH2
      .
      360      370      380      390      400      410      420
      .      .      .      .      .      .      .
      TTATTTGAAC TAACCAATCA GTTGGCTTCT CGCTTCTGTT CGCGCGCTTC CGCTCTCCGA CCTCAATAAA
      AATAAACTTG ATTGCTTAGT CAAGCGAAGA CGGAAGACAA CGCGCGCAAG CGCAGAGGCT CGAGTTATTT

      >Aap718
      .
      >Bam1
      .
      >Sma1
      .
      >Ava1
      .
      >Acl1
      .
      >Rsa1
      .
      >Ban2
      .
      >BssH2
      .
      >Hga1
      .
      >Tth1111
      .
      >Xma1
      .
      >Kpn1
      .
      430      440      450      460      470      480      490
      .      .      .      .      .      .      .
      AGAGCCCAACA ACCCTCACT CGGCGGCCA GTCTTCGGAT AGACTGCGTC GCGCGGGTAC CGTATTCCCA
      TCTCGGGTGT TCGCGAGTGA GCGCGCGGT CAGAAGGCTA TCTGACGCAG CCGCGCCCATG GGCATAAGGG

      >Sty1
      .
      500      510      520      530      540      550      560
      .      .      .      .      .      .      .
      AATAAAGCCT CTTGCTGTTT GCATCCGAAT CGTGGTCTCG CTGTTCTCTG CGAGGGTCTC CTCTGAGTGA
      TATTTTCCGA GAACGACAAA CGTAGGCTTA GCACGAGAGC GACAAGGAAC CCTCCGAGAG GAGACTCACT

      >Ban2
      .
      570      580      590      600      610      620      630
      .      .      .      .      .      .      .
      TTGACTACCC ACGACGGCGG TCTTTCATTT CGCGGCTGTT CGCGGATTTC GAGACCGCTG CCCAGGGACC
      AACTGATGGG TGCTGCCCCC AGAAGTAAA CCGCGGAGCA GCGCCTAAGC CTCTCGGGAC CGGTCCCTGG

      >Bcl1
      .

```

>Cfr1  
 .  
 >Eae1  
 .  
 >extended\_packing\_signal  
 .  
 640 650 660 670 680 690 700  
 . . . . .  
 ACCGACCCAC CACCGGGAGG TAAGCTGGCC ACCAACTTAT CTGTGTCTGT CCGATTGTCT AGTGTCTATG  
 TGGCTGGGTG GTGGCCCTCC ATTGCACCGG TCGTTCAATA CACACAGACA GGCTAACAGA TCACAGATAC

>Spe1  
 .  
 >Hga1  
 .  
 710 720 730 740 750 760 770  
 . . . . .  
 TTTGATGTTA TCGCCCTCCG TCTGTACTAG TTAGCTAACT AGCTCTGTAT CTGCGCCGACC CGTGTCTGAA  
 AACTACAAT ACGCGGAGCC ACACATGATC AATCGATTGA TCGACACATA GACCGCCTCG GCACACCTT

>Eco521  
 .  
 >Cfr1  
 .  
 >Xma3  
 .  
 >Eag1  
 .  
 >Eae1  
 .  
 780 790 800 810 820 830 840  
 . . . . .  
 CTGACGAGTT CTGAACACCC GCGGCCAACC CTGCGAGACC TCCACGGGAC TTGCGGGGCC GTTTTGTGG  
 GACTOCTCAA GACTTGTGGG CCGGCGTTGG GACCTCTCTC AGGCTCCTCT AAGCCCCCGG CAAAAACAC

>EcoK1  
 .  
 >Bsu36I  
 .  
 >Aoc1  
 .  
 >Sma1  
 .  
 >Eco81I  
 .

Table 6 (Cont'd)

```

>Cvn1
  |
>Hst2
  | 850
  |
  | 860
  |
  | 870
  |
  | 880
  |
  | 890
  |
  | 900
  |
  | 910
  |
CCCCACCTGA GGAAGGGAGT CGATGTGGA TCCGACCCCG TCAGGATATG TGGTTCTGGT AGGAGACGAG
GGGCTGGACT CCTTCCCTCA GCTACACCTT AGGCTGGCGC AGTCCTATAC ACCAAGACCA TCCTCTGCTC

>Hga1
  |
  | 920
  |
  | 930
  |
  | 940
  |
  | 950
  |
  | 960
  |
  | 970
  |
  | 980
  |
AACCTAAAAC AGTTCCCGCG TCCGTCTGAA TTTTTCCTTT CCGTTTGGAA CCGAAGCCCG CCGTCTTGTG
TTGGATTTTG TCAAGCGCGG AGGCAGACTT AAAAAGGAAA GCGAAACCTT CCGTTCCCGG CCGAGAACAG

>Pst1
  |
  | 990
  |
  | 1000
  |
  | 1010
  |
  | 1020
  |
  | 1030
  |
  | 1040
  |
  | 1050
  |
TGCTGCAGCA TCGTTCTGTG TTCTCTCTGT CTCACTGTCT TTCTGTATTT GTCTGAAAT TAGCGCCAGA
ACGACGTCGT AGCAAGACAC AACAGAGACA GACTGACACA AAGACATAAA CAGACTTTTA ATCCCGGTCT

```

```

>Aoc1
  |
>Sau1
  |
>Cvn1
  |
>Hst2
  |
>Bsu361
  |
>Afl2
  |
  | 1060
  |
  | 1070
  |
  | 1080
  |
  | 1090
  |
  | 1100
  |
  | 1110
  |
  | 1120
  |
CTGTTACCA TCCCTTAAAT TTGACCTTAG GTCAGTGGAA AGATGTGAG CCGATGGCTC ACAAGCAGTC
GACAATGCTG ACCGAATTCA AACTCGAATC CAGTGACCTT TCTACAGCTC GCGTAGCGAG TGTTCGCTAG

```

```

>Cfr1
  |
>AapA1
  |
>Ea 1
  |
>Eae1
  |

```

Table 6 (Cont'd)

	>Hae2	>BstE2		>Pst1	>Bcl1	>Hae2	>Cfr1
1130	1140	1150	1160	1170	1180	1190	
CGTAGATGTC	AAGAAGAGAC	GTTGGGTTAC	CTTCTGCTCT	GCAGAATGCC	CAACCTTTAA	CGTGGGATCG	
CCATCTACAG	TTCTTCTCTG	CAACCCAATG	GAAGACGAGA	CGTCTTACCG	GTTGGAAATT	GCAGCCTACC	
	>Bam1	>Hph1		>Hph1			
1200	1210	1220	1230	1240	1250	1260	
CGCGAGAGCG	GCACCTTTAA	CGAGACCTC	ATCAGCCAGG	TTAAGATCAA	GGTCTTTTCA	CCTGGGCGGC	
GGCGCTCTGC	CGTGGAAATT	GGCTCTGCAG	TAGTGGGTCC	AATTCTAGTT	CCAGAAAAGT	GGACCGGGCG	
	>Pss1						
	>Dra2						
	>EcoO109I						
	>PpuM1						
	>Tth111I			>Sty1			
1270	1280	1290	1300	1310	1320	1330	
ATGGACACCC	AGACCAGGTC	CCCTACATCG	TGACCTGGCA	AGCCTTGGCT	TTTGACCCCC	CTCCCTGGGT	
TACCTGTGGG	TCTGGTCCAG	CGCATGTAGC	ACTGGACCTT	TCCGAACCGA	AAACTGGGGG	GAGGGACCCA	
	>Rsa1						
1340	1350	1360	1370	1380	1390	1400	
CAAGCCCTTT	GTACACCTTA	AGCCTCGGGC	TCTCTTCTCT	CCATCGGGCC	CGTCTCTCCC	CCTTCAAGCT	
GTTGGGGAAA	CATGTGGGAT	TGGGAGGGCG	AGGACAAGCA	GCTAGCGGGC	GCAGACACCG	CGAATTGCA	
				>EcoN1	>EcoR1		
1410	1420	1430	1440	1450	1460	1470	
CGTGGTTCGA	CGCGGGCTCG	ATGCTGCTTT	TATCCAGGCC	TCACTGCTTC	TCTAGGCGGG	AATTCGTTAG	
CGAGCAAGCT	CGCGGGGAGC	TAGGAGCGGA	ATAGGTGGGG	AGTCAGGAAG	AGATCGGGCC	TTAAGCAATC	

>BspMI >RsaI  
 1480 1490 1500 1510 1520 1530 1540  
 CTTGGTAAGT GACCAGCTAC AGTCGGAAAC CATCAGCAAG CAGGTATGTA CTCTCCAGCG TGGGCCTCGC  
 GAACCATTC A CTGGTCGATG TCAGCCCTTTG GTAAGTCGTTT GTCCATACAT GACAGGTCGC ACCCGGACCG

[illegible]

1620                      1630                      1640                      1650                      1660                      1670                      1680  
 .                      .                      .                      .                      .                      .                      .  
 TCAACCGCTG ACTATCTTCC AGGTCAATTGT TCCAACATCG CCTGTGGAT CGACAGGATG CAACTCCTGT  
 GAGTTGGGAC TGATAGAACG TCCAGTAACA AGGTTGTACC GGGACACCTA GCTGTCTTAC GTTGAGGACA

>HglAI  
 >ApeLI  
 1690 1700 1710 1720 1730 1740 1750  
 CTTCATTGC ACTAAGTCTT GCACTTGTCA CAAACAGTGC ACCTACTTCA AGTTCTACAA AGAAACACA  
 CAACGTAACG TGATTACAAA CGTGAACAGT GTTTGTCAAG TCGATGAAGT TCAAGATGTT TCTTTTCTGT

```

>Pvu2
      |
>Ksp82      1760      1770      1780      1790      1800      1810      1820
      |      |      |      |      |      |      |
GCTGCAACTG GAGCATTAC TCTGCGATT ACAGATGATT TTGAATCGAA TTATATATTA CAGCAATGCG
CGACGTTGAC CTGCTAAATG ACGAAGTAAA TGTCTACTAA AACTTAAGCTT AATATATTAAT GTTCTTAGCG

```

```

      >SphI
      ▲
    >Ksp(7524)I
      ▲
    >KspHI
      ▲
1830   | 1840       1850       1860       1870       1880       1890
      ●   |   ●       ●       ●       ●       ●       ●
AAACTCAACC GCATGCTCAG ATTTAAGTTT TACATGCCCA AGAAGGCCAC AGAACTGAAA CATCTCCAGT
TTTGAGTCCG CGTACGAGTG TAAATTCAAA ATGTACCGGT TCTTCCCGTG TCTTCACTTT GTAGACGTCA

```

Table 6 (Cont'd)

<div>&gt;Xba1</div>							<div>&gt;Afl2</div>	
<div>↓</div>							<div>↓</div>	
<div>1900191019201930194019501960</div>								
<div>GTCTAGAAGAAGAACTCAAACTCTCTGCAAGAGCTGCTAAA</div>							<div>TTTAGCTCAAAGCAAAAAGTTCACTTTAAG</div>	
<div>CAGATCTTCTTCTTGAGTTTGGACACCTCC</div>							<div>TTCAAGATTTAAATOCAGTTTCGTTTTTGAAGTGAATTC</div>	
<div>&gt;Ava1</div>								
<div>↓</div>								
<div>&gt;Aqr2</div>								
<div>↓</div>								
<div>&gt;Aql1</div>								
<div>↓</div>								
<div>&gt;Ccr1</div>								
<div>↓</div>								
<div>&gt;PaeR71</div>							<div>&gt;Xho2</div>	
<div>↓</div>							<div>↓</div>	
<div>&gt;Xho1</div>							<div>&gt;BstY1</div>	
<div>↓</div>							<div>↓</div>	
<div>1970198019902000201020202030</div>								
<div>GGCTAGGGGAC</div>							<div>TTAATCAGCAATATCAAGCT</div>	
<div>AAATAGTTCTC</div>							<div>GAGCTAAAGCGATCTGAAAC</div>	
<div>AACATTCATG</div>							<div>AAATTCATG</div>	
<div>CGGATCCCTG</div>							<div>AATTAGTGGTTATAGTTCCA</div>	
<div>TTATCAAGAC</div>							<div>CTCGATTTCCCTAGACTTTG</div>	
<div>TTCTAAGTAC</div>								
<div>&gt;BstX1</div>								
<div>↓</div>								
<div>2040205020602070208020902100</div>								
<div>TGTGAATATG</div>							<div>CTGATGAGACAGCCACCAAT</div>	
<div>GTGGAATTTT</div>							<div>TCAACAGATG</div>	
<div>GATTACCTTT</div>							<div>TGTCAAAGCA</div>	
<div>ACACTTATAC</div>							<div>GACTACTCTGTCGGTGGTAA</div>	
<div>CACCTTAAAG</div>							<div>ACTTGTCTACCTAATCGAAA</div>	
<div>ACAGTTTGGT</div>								
							<div>&gt;BamH1</div>	
							<div>↓</div>	
							<div>&gt;BstY1</div>	
							<div>↓</div>	
							<div>&gt;Xho2</div>	
							<div>&gt;HspB2</div>	
							<div>↓</div>	
							<div>&gt;simian_virus_40_early_promoter</div>	
							<div>↓</div>	
							<div>&gt;Mo-MuSV_DNA_end/simian_virus_40_DNA_start</div>	
							<div>↓</div>	
<div>2110212021302140215021602170</div>								
<div>TCATCTCAAC</div>							<div>ACTAACTTGA</div>	
<div>TAATTAAGTG</div>							<div>CTTCCCACTT</div>	
<div>AAAACATATC</div>							<div>AGGATCCGCT</div>	
<div>GTGGAATGTC</div>							<div>AGTACAGTTG</div>	
<div>TCATTGAACT</div>							<div>ATTAATTCAC</div>	
<div>GAAGCGTGAA</div>							<div>TTTTCTATAG</div>	
<div>TCCTAGCGCA</div>							<div>CACCTTACAC</div>	
							<div>&gt;BcoT22</div>	
							<div>↓</div>	
							<div>&gt;Hsi1</div>	
							<div>↓</div>	
							<div>&gt;Ava3</div>	

Table 6 (Cont'd)

>Nsp(7524)1  
 .  
 >NspH1  
 .  
 >Sph1  
 .

2180	2190	2200	2210	2220	2230	2240
TGTCAGTTAG	GGTGTGGAA	GTCCCCAGGC	TCCCCAGCAG	GCAGAAGTAT	GCAAAGCATG	CATCTCAATT
ACAGTCAATC	CCACACCTTT	CAGGGGTCCG	AGGGGTGGTC	CGTCTTCATA	CGTTTGGTAC	GTACAGTTAA

>Nsi1  
 .  
 >Ava3  
 .  
 >EcoT221  
 .  
 >Nsp(7524)1  
 .  
 >NspH1  
 .  
 >Sph1  
 .

2250	2260	2270	2280	2290	2300	2310
AGTCAGCAAC	CAGGTGTGGA	AAGTCCCCAG	GCTCCCCAGC	AGGCAGAAAT	ATGCAAAAGCA	TGCATCTCAA
TCAGTGGTTG	GTCCACACCT	TTACAGGGTC	CCAGGGGGTC	TCCGTCTTCA	TACGTTTGGT	ACGTAGAGTT

2320	2330	2340	2350	2360	2370	2380
TTAGTCAGCA	ACCATAGTCC	CGCCCTTAAC	TCCGGCCATC	CGGCCCTTAA	CTCCGGCCAG	TTCGGCCCAT
AATCAGTGGT	TGGTATCAGG	GCGGGGATTG	AGCGGGGTAG	GGCGGGGATT	GAGCGGGGTC	AAGCGGGGTA

>Nco1  
 .  
 >Sty1  
 .

>Sfi1  
 .  
 >Bgl1  
 .

2390	2400	2410	2420	2430	2440	2450
TCTCCGGCCC	ATGGCTGACT	AAATTTTTTT	ATTATGCCAG	AGGCCGAGGC	CGGCTCGGCC	TCTGAGCTAT
AGAGGCGGGG	TACCGACTCA	TTAAAAAATA	TAAATACGTC	TCCGGCTCCG	GCGGAGCCCG	AGACTCGATA

>Sty1  
 .  
 >Ava2  
 .  
 >Stu1  
 .

>BspH1  
 .

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Table 6 (Cont'd)

>AatI                      >Hind3                      >PstI  
 2460                      2470                      2480                      2490                      2500                      2510                      2520  
 TCCACAAGTA GTGAGGACGC TTTTTCGAG CCTAGGCTT TTGCAAAAAG CTTCGGCTCC AGGTCGACGC  
 ACGTCTTCAT CACTCCTCCG AAAAACCCTC CCGATCCGAA AACGTTTTTC GAACCCGACG TCCAGCTCCG  
  
 >BclI  
 >Xho2  
 >BstY1  
 Lmian\_virus\_DNA\_end/Tn5\_DNA\_start  
 2530                      2540                      2550                      2560                      2570                      2580  
 GGATCTGATC AACAGACAGG ATCAGGATCG TTTCGC ATG ATT CAA CAA CAT CCA TTG CAC CCA CGT TCT  
 CCTAGACTAG TTCTCTGTCC TACTCCTAGC AAAGCG TAC TAA CTT GTT CTA CCT AAC CTC CGT CCA AGA  
 Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser>

>EcoS2I  
 >EagI  
 >EaeI  
 >CfrI  
 >XmaI  
 2590                      2600                      2610                      2620                      2630                      2640                      2650  
 CCG GCC CGT TCG GTG CAG ACG CTA TTC CGC TAT GAC TCG CCA CAA CAG ACA ATC CGC TCG TCT  
 CGC CGC CCA ACC CAC CTC TCC GAT AAG CCG ATA CTG ACC CGT GTT CTC TGT TAG CCG ACG AGA  
 Pro Ala Ala Trp Val Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser>

>Hae2  
 >BbeI  
 >Hae1  
 >Acy1  
 >Aha2

2660 2670 2680 2690 2700 2710

GAT GCC GCG CTG TTC GCG CTG TCA GCG CAG GCG GCG GCG CTT CTT TTT GTC AAG ACC GAG CTG  
CTA CCG CCG CAC AAG GCC GAC AGT CCG CTC CCC GCG GCG CAA GAA AAA CAG TTC TCG CTC GAC  
Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe Val Lys Thr Asp Leu

2720 2730 2740 2750 2760 2770

TOC CGT GCC CTG AAT GAA CTG CAG GAC GAG GCA GCG GCG CTA TCG TCG CTG GCG ACG ACG GCG  
ACG CCA CCG GAC TTA CTT GAC CTC CTG CTC CGT CCG GCG GAT ACG ACC GAC CCG TCG TCG CCG  
Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly

2780 2790 2800 2810 2820 2830 2840

GTT CCG TCG CCA CCT GTC CTC GAC GTT GTC ACT GAA GCG GCA ACG GAC TCG CTG CTA TTC GCG  
CAA CCA ACG CGT CCA CAC GAG CTG CAA CAG TGA CTT CCG CCT TCG CTC ACC GAC GAT AAC CCG  
Val Pro Cys Ala Ala Val Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly

2850 2860 2870 2880 2890 2900

GAA TG CCG GCG CAG GAT CTC CTG TCA TCT CA CTT CCG CCT GCG GAG AAA GTA TCG ATC ATG  
CTT CAC GCG CCC CTC CTA GAG GAC AAT AGA GTG GAA CCA CCA CCG CTC TTT CAT ACG TAG TAC  
lu Val Pr Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys Val Ser Il Met

[illegible]

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      >Cfr101
      >Cfr1
      >Eae1
      >Hae1
      >Rsr2
3160      3170      3180      3190      3200      3210
GGC GGC TTT TCT GGA TTC ATC GAC TGT CCC GCG CTG GGT CTC GCG CAC GGC TAT CAG GAC ATA
CCG GCG AAA AGA CCT AAG TAG CTG ACA CCG CCC GAC CCA CAC GCG CTG GCG ATA GTC CTG TAT
Gly Arg Phe Ser Gly Phe Ile Asp Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile>

3220      3230      3240      3250      3260      3270      3280
GCG TTG GCT ACC CGT GAT ATT GGT GAA GAG CTT GCG GCG GAA TCG GCT GAC CCG TTC CTC GTC
CCG AAC CGA TCG GCA CTA TAA CGA CTT CTC GAA CCG CCG CTT ACC CGA CTG CCG AAG GAG CAC
Ala Leu Ala Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe Leu Val>

3290      3300      3310      3320      3330      3340
CTT TAC GGT ATC GCG GCT CCC GAT TCG CAG CCG ATC GCG TTC TAT CCG CTT CTT GAC GAG TTC
GAA ATG CCA TAG CCG CGA GCG CTA AGC GTC GCG TAG CCG AAG ATA CCG GAA GAA CTG CTC AAG
Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe Tyr Arg Leu Leu Asp Glu Phe>

>Ple1
>Tn5_DNA_end/_Mo-MuLV_DNA_start
3350      3360      3370      3380      3390      3400      3410      3420
TTC TGA CCGGGACTC TCGCGTTCCA TAAAATAAAA GATTTTATTT AGTCTCCAGA AAAAGCGCGCG AATCAAGAC
AAG ACT CGCCCTGAG ACCCCAGCT ATTTTATTTT CTAAAATAAA TCAGAGCTCT TTTTCCCGCC TTACTTTCTG
Phe End>

>Afl2
>Khe1
3430      3440      3450      3460      3470      3480      3490
CCACCTGTA GGTTCGGCA GCTAGCTTAA GTAAAGCCAT TTGCAAGGC ATCGAAAAAT ACATAACTGA
GGGTGGACAT CCAAAACGCT CGATCGAAT CATTCGGTA AAACGTTCCG TACCTTTTA TGTATGACT

>HspB2
>Pvu2
>EcoR5
3500      3510      3520      3530      3540      3550      3560
GAATAGAGAA GTTCAGATCA AGGTACGAA CAGATCGAAG AGCTGAATAT GCGCCAAACA GCATATCTGT
CTTATCTCTT CAAGTCTAGT TCCAGTCCTT GTCTACCTTG TCGACTTATA CCGCGTTGT CCTATAGACA

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>Bae2      >Bae1
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>Eae2      >Aep718
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>Ebe1      >Pie1
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Table 6 (Cont'd)

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Table 6 (Cont'd)

↓ 4200 4210 4220 4230 4240 4250 4260  
 ↓ ↓ ↓ ↓ ↓ ↓ ↓  
 AGCGCGTGT CCCTCGTCTC CCGCGCGCAG CATGACCCAG TCACGTAGCG ATACCGCAGT GTATACTGGC  
 TCGCCACAA CCGCCACAG CCGCGCGTGC GTACTCGGTC AGTCATCGC TATCGCCTCA CATATGACCG

>HglA1  
 ↓  
 >RsaI >ApaI >NdeI  
 ↓ ↓ ↓  
 4270 4280 4290 4300 4310 4320 4330  
 ↓ ↓ ↓ ↓ ↓ ↓ ↓  
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>HaeII >PleI  
 ↓ ↓  
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 ↓ ↓ ↓ ↓ ↓ ↓ ↓  
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 GCATTCTCT TTTATCGCGT AGTCGGGAG AAGGCGAAGG AGCGAGTCAC TCAGCGACCG CAGCCACCAA

4410 4420 4430 4440 4450 4460 4470  
 ↓ ↓ ↓ ↓ ↓ ↓ ↓  
 CGCGTGGCG GAGCGGTATC AGCTCACTCA AAGCGCGTAA TACCGTTATC CACAGATCA GGGGATAAGC  
 GCGGACCGCG CTGCGCATAG TCGAGTGAGT TTCCGCCATT ATGCCAATAG GTGTCTTAGT CCGCTATTGC

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 ↓  
 >HspH1  
 ↓  
 >AflII  
 ↓  
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 CAGGAAAGAA CATGTGACCA AAAGCGCAGC AAAGCGCAG CAACCGTAAA AAGCGCGCGT TGCTGGCGTT  
 GTCTTTTCTT GTACACTCGT TTTCCGGTGC TTTCCGGTGC CTTCGCATT TTTCCGGCGCA ACGACCGCAA

>HgaI  
 ↓  
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 ↓ ↓ ↓ ↓ ↓ ↓ ↓

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Table 6 (Cont'd)

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 AAAGGTATCC CAGCGCCCCG CACTGCTCGT AGTGTTTTTA CTGGGAGTT CAGTCTCCAG CGCTTTGGCG

4620 4630 4640 4650 4660 4670 4680  
 ACAGGACTAT AAAGATACCA GCGGTTTCCC CCTGGAAGCT CCTCGTGGG CTCTCTGTT CCGACCCCTGC  
 TGTCCTGATA TTCTATGCT CCGAAAGCG CGACCTTCCA GCGAGCACCG CACAGGACAA CGCTCGGACG

&gt;Hae2

4690 4700 4710 4720 4730 4740 4750  
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 GCGAATGGCC TATGGACAGG CCGAAAGAGG GAAGCCCTTC GCACCGCGAA ACAGTATCGA GTGGACATC

&gt;HglAI

&gt;ApaLI

4760 4770 4780 4790 4800 4810 4820  
 GTATCTCAGT TCGGTGTAGG TCGTTGGCTC CAAGCTGGGC TGTGTCCAGG AACCCCGGT TCAGCCCGAC  
 CATAGAGTCA AGCCACATCC AGCAAGCGAG GTTCGACCCG ACACACGTGC TTGGGGGGCA AGTCGGGCTG

&gt;NspB2

&gt;PleI

4830 4840 4850 4860 4870 4880 4890  
 CGCTCGGCTT TATCGGTAA CTATGCTCTT GAGTCCAAAC CGGTAAGACA CGACTTATCG CCACTGGCAG  
 GCGACGCGCA ATAGCCCATT GATACCAGAA CTCAGGTTCC GCCATTCTGT CCTCAATAGC GGTGACCGTC

&gt;AluNI

4900 4910 4920 4930 4940 4950 4960  
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 GTGGGTGACC ATTGTCTTAA TGGTCTGGT CCATACATCC GCCACGATGT CTCAGAACT TCACACCGG

4970 4980 4990 5000 5010 5020 5030  
 TAACTACCGC TAACTACGAA CGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGAAAA  
 ATTGATGCGG ATGTGATCTT CCGTCTATAA ACCATAGACG CGAGACGACT TGGTCAATG GAAAGCTTTT

&gt;NspB2

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Table 6 (Cont'd)

PCT/US92/08999

5040 5050 5060 5070 5080 5090 5100  
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>Xho2 >BstY1  
 >BstY1 >Xho2 >Hga1  
 5110 5120 5130 5140 5150 5160 5170  
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>BstY1  
 >Xho2 >BstY1 >Dra1  
 >Hae2 >BspH1 >Hph1 >Xho2 >Aha3  
 5180 5190 5200 5210 5220 5230 5240  
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 CTTGCTTTTG AGTCAATTC CCTAAAACCA GTACTCTAAT AGTTTTTCCT ACAAGTGGAT CTAGGAAAAAT

>Dra1  
 >Aha3  
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>Bam1 >Ple1  
 5320 5330 5340 5350 5360 5370 5380  
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 ATTAGTCACT CCGTGGATAG AGTCGCTAGA CAGATAAAGC AAGTAGGTAT CAACCGACTG ACGGGCAGCA

>Bph1  
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 CATCTATTGA TGCTATGCCC TCCCGAATGG TAGACGGGGC TCAACGAGTT ACTATGGGCG TCTCGGTGGG

>Cfr101 >Bgl1  
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[illegible]

>Hinc2  
 >Hind2  
 >Acy1  
 >Hga1  
 >Aha2  
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 ACTCTTATCA CATACGCGCG TCGCTCAACG AGAACCGGCG GCAGTTGTCC CCTATTATCG CGCGGTGTAT  
  
 >Aap700  
 >Aha3  
 >Dra1  
 >Hga1  
 >Xba1  
 >Hae2  
 >BstY1  
 >Xho2  
 >HepB2  
 5950 5960 5970 5980 5990 6000 6010  
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 >Xho2  
 >BstY1  
 >Hga1  
 >Apa1  
 >Bph1  
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 >Bph1  
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 GTTCTGCGGT GAGCAAAAC AGGAAGCCAA AATGCGGCAA AAAAGCGCAAT AAGGGCGACA CCGAAATGTT  
 CAAAGACCCA CTGGTTTTTG TCCTTCCGTT TTACCGCGTT TTTTCCCTTA TTCCCGCTGT GCGTTTACAA  
  
 >Sap1  
 >BspH1  
 6160 6170 6180 6190 6200 6210 6220

Table 6 (Cont'd)

GAATACTCAT ACTCTTCCCTT TTTCATATATT ATTCAAGCAT TTATCAGGGT TATTGCTCTCA TGAGCCGATA  
CTTATCGAGTA TCAGAAGGAA AAAGTTATAA TAACTTGGTA AATAGTCCCA ATAACAGAGT ACTCGCCTAT

6230 6240 6250 6260 6270 6280 6290  
CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCCACAT TTCCCCGAAA AGTGCCACCT  
GTATAAACTT ACATAAATCT TTTTATTTGT TTATCCCCAA GCGCGGTGTA AAGCGGCTTT TCACGGTCCA

>Aat2  
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>Aha2  
|  
>Acy1  
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>Hae2  
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6300 6310 6320 6330 6340 6350 6360  
GACGTCTAAG AAACCATAT TATCATGACA TTAAGCTATA AAAATAGGCG TATCAAGAGG CCCTTTGGTC  
CTGCAGATTC TTTCGTAATA ATAGTACTGT AATTCGATAT TTTTATCCGC ATAGTCTCTC GCGAAAGCAG  
TTCAA  
AAGTT

>BspH1  
|  
>Pae1  
|  
>EcoO109I  
|  
>Dra2  
|

zymes which do not cut L15NRIIL2 :

Ace3	Bgl2	Cla1	Hpa1	Hru1	SnaB1
Apa1	Bsm1	Dra3	Hlu1	PflM1	Spl1
Aru2	BspM2	Eco47III	Hro1	Sac2	Sst2
Ban3	BstB1	Esp1	Not1	Sal1	

To generate the LXSN-RI-IL2 retroviral vector, 10 micrograms of pLXSN-RI-IL2 DNA was transfected into the ecotropic packaging cell line PE501 by standard calcium phosphate precipitation methods (Miller et al., Mol. Cell Biol. 6:2895, 1986). The transfected PE501 cell line was grown in DMEM medium with 10% FCS. The medium was changed after 24 hours and supernatant harvested 24 hours later to infect the amphotropic packaging cell line PA317 as described (Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989). The infected PA317 cells were harvested by trypsinization 24 hours later and replated 1:20 in DMEM containing 10% FCS and the neomycin analogue G418 (400 µg/ml). The cells were grown at 37°C in 7% CO<sub>2</sub> atmosphere. The selection medium was changed every 5 days until colonies appeared. On day 14, twenty colonies were selected, expanded and tested for viral production by standard methods (Xu et al., Virology 171:331-341, 1989). Briefly, supernatants were harvested from confluent culture dishes, passed through a .45 µm filter, diluted with DMEM with 10% FCS and utilized to infect NIH 3T3 cells in the presence of 8 µg/ml polybrene. After 24 hours, the infected NIH 3T3 cells were grown in culture medium that contained the neomycin analogue G418. After 12-14 days, the colonies were stained, counted and the viral titer calculated as described (Xu et al., Virology 171:331-341, 1989).

Colonies with the highest viral titers ( $>10^4$  infectious units/ml) were tested for IL-2 expression by Northern blot analyses. Colonies with the highest viral titers and documented IL-2 expression were cryopreserved and will be utilized as stock cultures to produce the LXSN-RI-IL2 retroviral vector trial.

EXAMPLE IVRETROVIRAL VECTOR CONSTRUCTION AND CYTOKINE EXPRESSION

To increase IL-2 production by transduced cell lines, vectors were used containing different promoters to drive IL-2 expression, and a human IL-2 cDNA was directionally sub-cloned into the insulin secretory signal peptide (17). The IL-2 cDNA was directionally sub-cloned into the parental plasmids of the LXS<sub>N</sub> (LTR promoter) and LNCX (CMV promoter) vectors (gifts of Dr. A.D. Miller) (18). The newly constructed vectors (Figure 1), designated as LXS<sub>N</sub>-IL2 and LNCX-IL2, were packaged in the PA317 cell line for production of retroviral supernatant. As a control, the high level expressing, double copy vector DC/TKIL-2 vector (thymidine kinase promoter) (a gift of Dr. E. Gilboa) was used for comparison.

These vectors were used to transduce a number of murine and human, primary and established cell lines. Pools of transduced cells were selected and expanded in DMEM medium, containing 10% fetal bovine serum (FBS) and 400 µg/ml of active G-418, a neomycin analogue. The results of expression studies in the MCR9 and Balb/c 3T3 cell lines are presented in Table 7.

Table 7

Comparison of IL-2 expression by fibroblasts transduced with different IL-2 vectors.				
5	Fibroblast	Vector	<u>ng IL-2</u>	<u>Units IL-2</u>
			per 10 <sup>6</sup> cells per day	
10	Murine	LNCX (Control)	0.4 ±50%	<1
		LNCX-IL2	33.7 ±11%	67
		LXSN-IL2	6.6 ± 6%	13
		DC/TKIL-2	1.9 ± 5%	4
15	Human	LXSN (Control)	0.7 ±29%	1
		LNCX-IL2	159.5 ±17%	319
		LXSN-IL2	25.5 ±15%	51
		DC/TKIL-2	3.0 ±10%	6

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EXAMPLE VFIBROBLAST CULTURE AND CONDITIONS FOR RETROVIRAL  
TRANSDUCTION

The culture conditions for the growth of primary  
5 fibroblasts retroviral transduction were optimized.  
Primary fibroblasts were successfully cultured. The  
optimal conditions enable the growth of approximately  $3-4$   
 $\times 10^6$  primary fibroblasts from a  $12 \text{ mm}^2$  skin biopsy in  
approximately 4-6 weeks. Retroviral infection, G418  
10 selection, and expansion of the genetically modified  
fibroblasts takes an additional 4-6 weeks.

Exploring the conditions for genetic modification  
of primary fibroblasts suggests that optimal transduction  
may be obtained by the following procedure: The fibroblasts  
15 are synchronized in G1 phase by serum starvation, followed  
by stimulation with medium containing 15% fetal bovine  
serum 15 hours prior to transduction. The cells are then  
subjected to 2 cycles of retrovirus infection, each cycle  
lasting approximately 3 hours. The cells are refed with  
20 fresh media overnight, and then selection in G418 is  
initiated the next day. This method is capable of  
transducing 5-15% of the fibroblasts in a culture,  
depending on the multiplicity of infection.

This procedure was used to transduce a large  
25 number of primary and established fibroblasts. As an  
example, Table 8 compares the expression levels of IL-2 in  
fibroblast lines transduced with LXS-N-IL2.

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Table 8

Expression of IL-2 by fibroblasts transduced with LXS<sub>N</sub>-IL2.

5	Fibroblast Line	Species	Origin	<u>ng IL-2 Units IL-2</u>	
				per 10 <sup>6</sup> cells	per day
	Balb/c 3T3	Murine	Transformed	6.6 ± 6%	13
	MCR9	Human	Embryonic	25.5 ± 15%	51
10	NHDF 313	Human	Skin	25.0 ± 10%	50
	GT1	Human	Skin	15.0 ± 5%	30

These results indicate that the IL-2 expression levels in established, embryonic, and primary fibroblast cultures are similar. Comparison of these data with Table 7 suggest that IL-2 expression is affected more by factors such as different promoters than by the fibroblast line used. Similarly, changes in culture conditions can have important effects on IL-2 expression. Table 9 shows that transduced GT1 cells, a primary human fibroblast culture expressed 15-fold more IL-2 under 100 µg/ml G418 selection than under 25 µg/ml G418 selection. Several other primary fibroblast lines have also been transduced with our vectors and are currently growing under G418 selection.

Table 9

Effect of G418 concentration on IL-2 expression by GT1 cells transduced with LXSNI-IL2.	
5	
	Selection dose of G418
	ng IL-2 secreted per 10 <sup>6</sup> cells per day*
	25 µg/ml
10	50 µg/ml
	100 µg/ml
	1.0 ± 10%
	3.0 ± 6%
	15.0 ± 5%

\*After three weeks of G418 selection.

#### EXAMPLE VI

#### 15 COMPARISON OF IL-2 EXPRESSION LEVELS INDUCED PERIPHERAL BLOOD LYMPHOCYTES AND GENETICALLY MODIFIED FIBROBLASTS

In order to compare the production of IL-2 by genetically modified fibroblasts to that achieved by stimulating normal human peripheral blood lymphocytes (nPBIL) in vitro, nPBIL were isolated by Ficoll-Paque density centrifugation, and cultured in the presence of allogeneic nPBIL (mixed lymphocyte culture, MLC) or 2 µM calcium ionophore (CI) (A23187) free acid plus 17 nM phorbol 12-myristate 13-acetate (PMA). The results of this experiment, present in Table 10, indicate that the level of IL-2 expression in the PMA/CI stimulated normal T cell population was 2 ng/10<sup>6</sup> cells/24 hours. This is equivalent to IL-2 expression by Balb/c 3T3 fibroblasts transduced with DC/TKIL-2 (Table 7), our least productive vector. The level of IL-2 expression in the MLC was 130 pg/10<sup>6</sup> cells/24 hours. This was lower than the PMA/CI stimulated culture, presumably because PMA/CI induced a nonspecific response

while MLC resulted in specific Th stimulation. When the estimated percentage of antigen-specific Th in the MLC-stimulated population is taken into consideration, the level of IL-2 expression per stimulated T cell becomes equivalent for both methods.

Table 10  
Levels of IL-2 secretion by different cells.

10	Cells	pg IL-2 secreted per 10 <sup>6</sup> cells per day
	Lymphocytes:	
	Control (non-activated)	5 ± 50%
	PMA + Calcium Ionophore	2,000 ± 6%
15	Mixed lymphocyte culture	130 ± 90%
	Transduced fibroblasts:	
	MCR9-LXSN-IL2	24,000 ± 5%
	MCR9-LNCX-IL2	162,000 ± 20%
20	MCR9-DC/TKIL-2	10,000 ± 6%

#### EXAMPLE VII

#### FIBROBLAST MEDIATED CYTOKINE GENE THERAPY IN MURINE TUMOR MODELS

Two experimental protocols were used to study the efficacy of fibroblast-mediated cytokine gene therapy on induction of anti-tumor immunity. The first protocol was designed to test the effects of genetically modified fibroblasts on tumor implantation, while the second protocol was designed to induce a systemic anti-tumor immunity. The results of each experiment are presented with two figures and one table. In the first figure, the rate of tumor growth for each treatment group is presented

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as the mean tumor size in the group over time. In the second figure, a Kaplan-Meier curve presents the time of tumor onset for the individual animals in each treatment group. The number of animals, the number and percentage of tumor free animals, and the tumor size distribution patterns for each experiment are presented in a table.

EXAMPLE VII(a)

EFFECT OF FIBROBLAST MEDIATED CYTOKINE GENE  
THERAPY ON TUMOR IMPLANTATION

10 Mice were injected subcutaneously with mixtures  
of  $5 \times 10^4$  CT26 cells and  $2 \times 10^6$  fibroblasts genetically  
modified by different retroviral vectors to express IL-2.  
In the control arms injected with tumor cells only, or with  
tumor cells mixed with unmodified fibroblasts, 31 of 33  
15 animals (94%) developed tumors by 4 weeks (Figures 6 and 7,  
Table 9). In contrast, 22 out of the 34 animals (65%)  
receiving fibroblast mediated cytokine gene therapy were  
tumor free at 3 weeks, and 5 animals (18%) remain tumor  
free after 12 weeks. Those animals that received  
20 fibroblast mediated IL-2 therapy and developed tumor were  
characterized by a delayed onset and rate of tumor growth.

Table 11

Effect of IL-2 modified fibroblasts on tumor establishment and development.  
2 X 10<sup>6</sup> fibroblasts mixed with 5 X 10<sup>4</sup> CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number			Percent Tumor-free				Tumor Size (mm <sup>2</sup> )				Median Tumor Size (mm <sup>2</sup> )
	Total	Tumor-free	Tumor-bearing	Total	Tumor-free	Tumor-bearing	Percent Tumor-free	25-100	101-200	201-300	> 301	
After 12 Weeks:												
Control (no fibroblasts)	11	0	11		0%			1	0	1	9	420 ± 145
Unmodified fibroblasts**	13	2	11		15%			1	0	1	7	388 ± 265
DCTK-IL2 fibroblasts	13	0	13		0%			1	3	5	4	267 ± 168
LNCX-IL2 fibroblasts	13	5	8		39%			5	2	0	1	72 ± 90

\* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

\*\* Two mice in this arm developed intraperitoneal tumors which were not measurable.

After 3 weeks the mean tumor size (measured as the product of the longest and widest tumor axes) in the control group of mice was 128 mm<sup>2</sup>, compared to 68 and 7 mm<sup>2</sup> in groups of mice injected with tumor cells mixed with  
5 fibroblasts transduced with DC/TKIL-2 or LNCX-IL2, respectively. This resulted in a highly significant difference (corrected  $\chi^2 = 18.69$ ,  $p = 0.001$ ) between the IL-2 treated animals compared to the mice treated with CT26 alone or CT26 mixed with unmodified fibroblasts. After  
10 four weeks the equivalent measurements were 373,300 and 72 mm<sup>2</sup> (Table 11). It is notable that LNCX-IL2, the highest expressing vector caused substantially greater inhibition of tumorigenicity than the lower expressing vector DC/TKIL-2. A multivariate non-parametric statistical procedure  
15 (19,20), utilized to evaluate differences in tumor growth, demonstrated that after 4 weeks the differences between the growth curves for the four groups presented in Figure 2 were highly significant ( $p < 0.001$ ). Subsequent comparisons between the control arm and animals that  
20 received tumor cells mixed with IL-2 transduced fibroblasts revealed a significant difference ( $P < 0.05$ ). The differences between the animals injected with tumor cells alone, and those injected with tumor cells plus unmodified fibroblasts were not significant, while the differences  
25 between animals receiving low IL-2 expressing fibroblast, and those receiving high IL-2 expressing fibroblasts was significant ( $P = 0.05$ ).

When mice were injected with  $2 \times 10^6$  modified fibroblasts mixed with  $1 \times 10^5$  live tumor cells the results  
30 became more striking (see Figures 8 and 9, and Table 12). All the control animals developed tumors after 4 weeks whereas 33% and 27% of the animals treated with fibroblasts modified with the DCTK-IL2 or LXS-IL2 vectors (respectively) remain tumor free after 7 weeks (the  
35 experiment is ongoing). More dramatically, 75% of the animals treated with fibroblasts modified with the high st

IL-2 producing vector, LNCX-IL2, remain tumor free after 7 weeks. These data clearly demonstrate the importance of an initial high dose of IL-2 to prevent tumor establishment.

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Table 12

Effect of IL-2 modified fibroblasts on tumor establishment and development.  
2 X 10<sup>6</sup> fibroblasts mixed with 1 X 10<sup>5</sup> CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number		Percent Tumor-free	Tumor Size (mm <sup>2</sup> )				Mean Tumor Size (mm <sup>2</sup> )
	Total	Tumor- free		25-100	101-200	201-300	>301	
After 6 Weeks:*								
Control (no fibroblasts)**	13	0	13	0	5	2	5	315 ± 197
Unmodified fibroblasts**	20	0	20	0	2	3	14	350 ± 100
DCTK-IL2 fibroblasts	12	4	8	0	1	4	3	185 ± 141
LXSN-IL2 fibroblasts***	15	4	11	0	5	1	2	135 ± 121
LNCX-IL2 fibroblasts	8	6	2	2	0	0	0	8 ± 14

\* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

\*\* One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

\*\*\* Three mice in this arm developed intraperitoneal tumors which were not measurable.

As an additional control, mice were injected with CT26 cells genetically modified to express IL-2 (results not shown). Injection of up to  $1 \times 10^6$  IL-2 expressing tumor cells into Balb/c mice failed to produce tumors.

5 Injection of higher numbers however, resulted in some animals developing tumors with delayed onset. These data confirm the results reported in the literature (1). In order to compare the efficacy of IL-2 producing fibroblasts to IL-2 producing tumor cells, we mixed  $2 \times 10^6$  CT26 tumor

10 cells modified with the DCTK-IL2 vector with  $1 \times 10^5$  unmodified tumor cells. Figures 10 and 11, and Table 13 show that DCTK-IL2 modified tumor cells are somewhat effective in preventing tumor development. Four weeks after injection, the mean tumor size for the treatment arm

15 is  $303 \text{ mm}^2$ , compared to  $620 \text{ mm}^2$  for the control arm. After 22 weeks, one animal (10%) remains tumor free, compared to none in the control arms. Data for animals treated under the same conditions with DCTK-IL2 modified fibroblasts in a separate experiment are included for comparison purposes.

20 This comparison suggests that DCTK-IL2 modified tumor cells have an effect on tumor establishment similar to that of DCTK-IL2 modified fibroblasts.

Table 13

Effect of IL-2 modified cells on tumor establishment and development.  
 2 X 10<sup>6</sup> DCTK-IL2-modified CT26 tumor cells mixed with 1 X 10<sup>5</sup> CT26 cells compared to 2 X 10<sup>6</sup> DCTK-IL2-modified fibroblasts mixed with 1 X 10<sup>5</sup> CT26.

Cells mixed with tumor cells	Animal Number		Percent Tumor-free	Tumor Size (mm <sup>2</sup> )				Mean Tumor Size (mm <sup>2</sup> )
	Total	Tumor-free		25-100	101-200	201-300	>301	
After 22 Weeks:*								
Control (no fibroblasts)	5	0	5	0%	0	0	5	620 ± 190
Unmodified fibroblasts	5	0	5	0%	0	0	5	587 ± 69
DCTK-IL2-modified CT26 cells	10	1	9	10%	1	0	5	303 ± 179
DCTK-IL2-modified fibroblasts	8	2	6	25%	0	1	2	214 ± 158

\* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

EXAMPLE VII(b)EFFECT OF FIBROBLAST MEDIATE CYTOKINE GENE THERAPY  
ON SYSTEMIC ANTI-TUMOR IMMUNITY

Groups of Balb/c mice were immunized with  
5  $2.5 \times 10^5$  irradiated tumor cells either alone or mixed with  
 $2 \times 10^6$  transduced or unmodified fibroblasts, and challenged  
one week later with  $5 \times 10^4$  live tumor cells in the opposite  
flank. These results (Figures 12 and 13, and Table 14)  
demonstrate that immunization with irradiated tumor cells  
10 and transduced fibroblasts protect some animals against a  
live tumor challenge, but that the protection is only  
slightly better than that achieved by immunization with  
irradiated tumor cells alone or irradiated tumor cells  
mixed with unmodified fibroblasts.

Table 14

Effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity.  
Mice immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor cells 7 days prior to challenge with  $5 \times 10^4$  fresh tumor cells.

Fibroblasts mixed with irradiated tumor cells	Animal Number		Percent Tumor-free	Tumor Size (mm <sup>2</sup> )			Mean Tumor Size (mm <sup>2</sup> )
	Total	Tumor-free		25-100	101-200	201-300 > 301	
After 22 Weeks:*							
Control (saline)	20	0	20	0	0	1 19	574 ± 160
Irradiated CT26 only**	16	5	11	2	1	2 5	250 ± 277
Irradiated CT26 mixed with unmodified fibroblasts	15	4	11	0	1	3 7	266 ± 199
DCTK-IL2 fibroblasts**	25	10	15	4	1	1 8	172 ± 194

\* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

\*\* One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

In a second protocol similar to the one described above, animals were challenged with fresh tumor cells two weeks following immunization with irradiated tumor cells mixed with fibroblasts. The results, shown in Figures 14 and 15, and in Table 15, demonstrate that DCTK-IL2 modified fibroblasts mixed with irradiated tumor cells confers superior protection to subsequent tumor challenge than irradiated tumor cells alone, irradiated tumor cells mixed with unmodified fibroblasts, or irradiated tumor cells mixed with LNCX-modified fibroblasts. After 7 weeks, seven of ten animals (70%) treated with DCTK-IL2 modified fibroblasts remain tumor free compared to only one third of the control animals. At four weeks, the mean tumor size of this group was 41 mm<sup>2</sup>, compared to 180, 170, and 140 mm<sup>2</sup> for the three control groups. Animals treated with LNCX-IL2 modified fibroblasts were also protected against subsequent tumor challenge, but the results were less striking. In this group, 54% of the animals remain tumor free and the mean tumor size for the group at four weeks was 86 mm<sup>2</sup>. The number of tumor free animals in the group treated with LXSX-IL2 modified fibroblasts was similar to the control groups, although the tumors were slightly delayed in their onset. A multivariate non-parametric statistical procedure (19, 20), utilized to evaluate differences in tumor onset, demonstrated that the differences for the six arms presented in Figure 15 were significant ( $p = 0.012$ ). It further showed that the saline control arm and the arms that received irradiated tumor cells alone or mixed with unmodified or LNCX vector modified fibroblasts formed a statistical group. A second, distinct statistical group was formed by the three arms that received IL-2 vector modified fibroblasts mixed with irradiated tumor cells. Subsequent comparisons between the saline injected control arm and animals that received tumor cells mixed with IL2 transduced fibroblasts revealed a significant difference for all vectors ( $p < 0.05$ ).

Table 15

Effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity.  
Mice immunized with  $2 \times 10^6$  fibroblasts mixed with  $2.5 \times 10^5$  irradiated CT26 tumor cells 14 days prior to challenge with  $5 \times 10^4$  fresh tumor cells.

Immunization by fibroblasts mixed with irradiated tumor cells	Animal Number Total      Tumor- free      bearing		Percent Tumor-free	Tumor Size (mm <sup>2</sup> )			Mean Tumor Size (mm <sup>2</sup> )
				25-100	101-200	>301	
After 7 Weeks:*	8	1	7	13%			
Control (saline)**					0	2	245 ± 173
Irradiated CT26 only	10	3	7	30%	0	2	180 ± 155
Irradiated CT26 mixed with unmodified fibroblasts	6	2	4	33%	0	2	170 ± 160
Irradiated CT26 mixed with LNCX-modified fibroblasts	10	3	7	30%	3	0	140 ± 142
Irradiated CT26 mixed with LNCX-IL2-modified fibroblasts	13	7	6	54%	1	3	86 ± 112
Irradiated CT26 mixed with LXSN-IL2-modified fibroblasts	12	4	8	33%	5	0	111 ± 145
Irradiated CT26 mixed with DCTK-IL2-modified fibroblasts	10	7	3	70%	1	2	41 ± 75

\* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

\*\* One mouse in this arm developed an intraperitoneal tumor which was not measurable.

These results demonstrate the feasibility of using genetically modified fibroblasts as a means of delivering cytokine gene therapy. In all experiments, the LNCX-L2 vector proved superior in preventing tumor establishment while the DCTK-IL2 vector was better in the induction of systemic protection against subsequent tumor challenges. These contrasting effects, although somewhat surprising, can be explained by the observation that the CMV promoter is turned off in vivo five days after implantation while the TK promoter remains active for a longer period of time. The implication of this finding is that to apply this method of gene therapy successfully we have to use promoters that result in high level, sustained expression of IL-2 in vivo in the transduced fibroblasts.

The data obtained from this research effort has important implications for all cytokines that have either direct or indirect anti-tumor effects. Furthermore, this data suggests that anti-tumor efficacy is IL-2 dose dependent. Hence, construction of vectors which result in higher levels of cytokine secretion will be a significant advance toward the application of this method of gene therapy.

Reference numbers in parenthesis in the above examples correspond to the following list of references and are incorporated herein by reference.

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Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention.

5 Accordingly, the invention is limited only by the following claims.

## WE CLAIM:

1. A method of treating cancer in a patient comprising the stimulation of that patient's immune response against the cancer by immunizing said patient at a site other than an active tumor site with a formulation comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.
2. The method of claim 1 wherein tumor cells previously isolated from said patient provide the tumor antigens.
3. The method of claim 1 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma-interferon.
4. The method of claim 3 wherein one cytokine gene is interleukin-2.
5. The method of claim 1 wherein at least one cytokine gene is transferred into cells to generate CE cells by recombinant methods.
6. The method of claim 5 wherein the cytokine gene is present in an expression vector.
7. The method of claim 6 wherein said expression vector additionally contains a suicide gene.
8. The method of claim 5 wherein the CE cells are generated from fibroblasts and antigen-presenting cells.

9. A method for enhancing a patient's immune response to a cancer comprising:

- a) isolating fibroblasts from said patient;
- 5 b) culturing said fibroblasts in vitro;
- c) transducing said fibroblasts with a retroviral expression vector containing the gene coding for IL-2 and a gene coding for a tumor antigen in a retroviral expression vector, to express said tumor antigen and to express and secrete said IL-2 by said fibroblasts; and
- 10
- d) immunizing said patient with said fibroblasts that express IL-2 at a level sufficient to enhance an immune response but low enough to avoid substantial systemic toxicity and that express said tumor antigen, at a site
- 15
- 20 other than an active tumor site.

10. The method of claim 9 wherein said fibroblasts are further modified to express a suicide gene.

11. A composition for increasing a patient's immune response to tumor antigens comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.

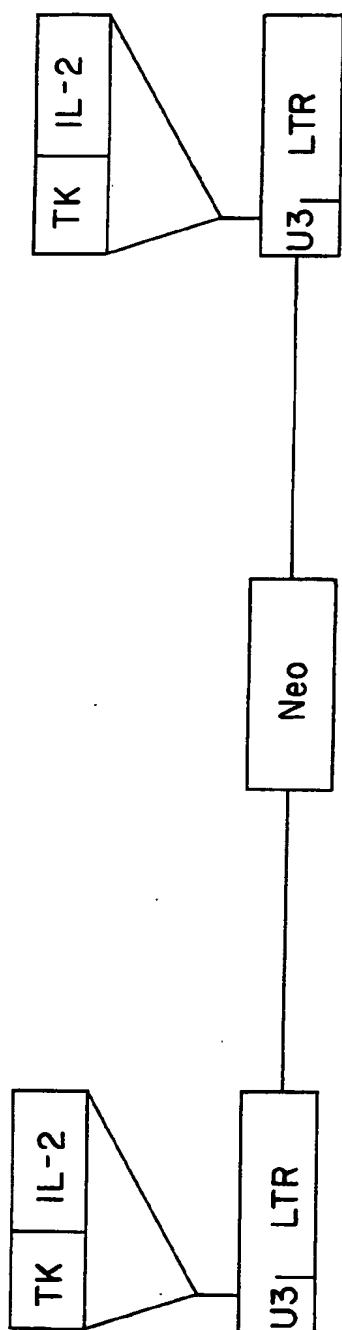
12. The composition of claim 11 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma interferon.

13. The composition of claim 12 wherein one cytokine gene is interleukin-2.

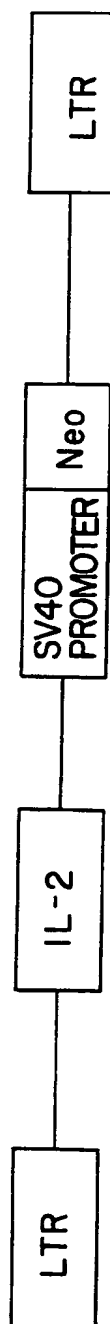
14. The composition of claim 11 wherein each cytokine gene is expressed at a level sufficient to stimulate the immune response but low enough to avoid substantial systemic toxicities.

15. The method of claim 9 wherein in said transducing step said retroviral expression vector has a promotor causing sustained secretion of IL-2.

16. The method of claim 15 wherein said retroviral expression vector causes the secretion of at least four units of IL-2 per day for a period of ten days or longer.



RETROVIRAL VECTOR DC/TKIL2



RETROVIRAL VECTOR LXSN-IL2



RETROVIRAL VECTOR LNCX-IL2

FIG. 1

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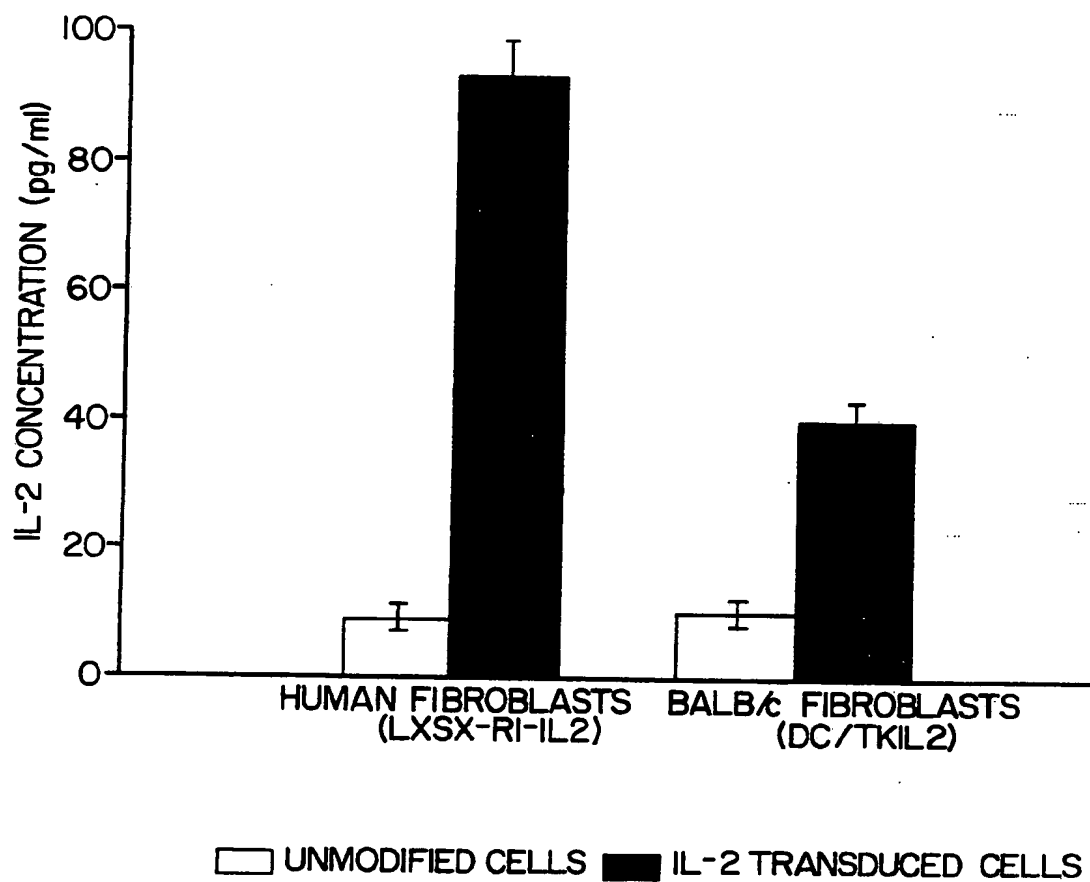


FIG. 2

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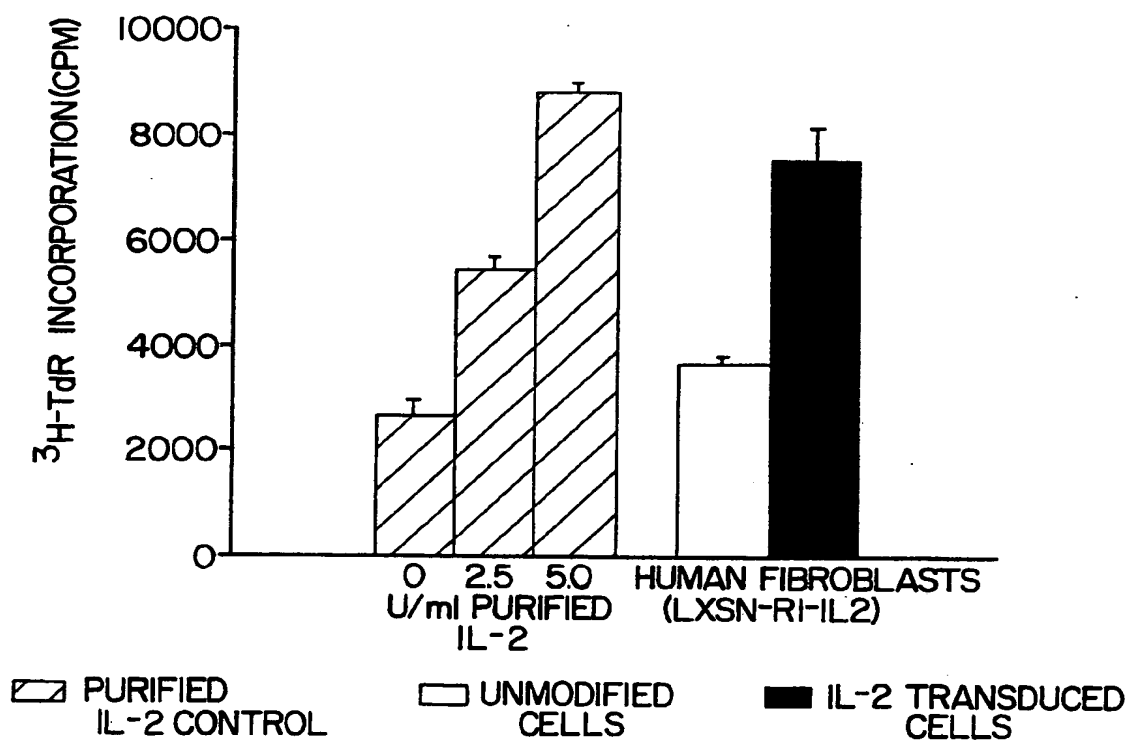


FIG. 3

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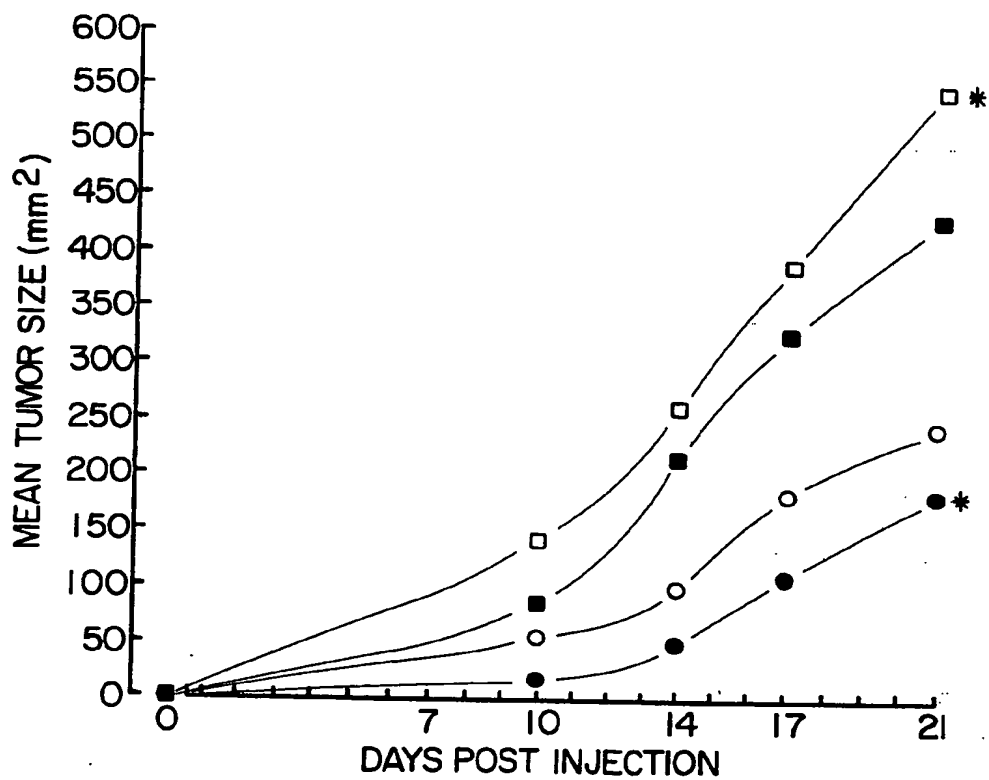
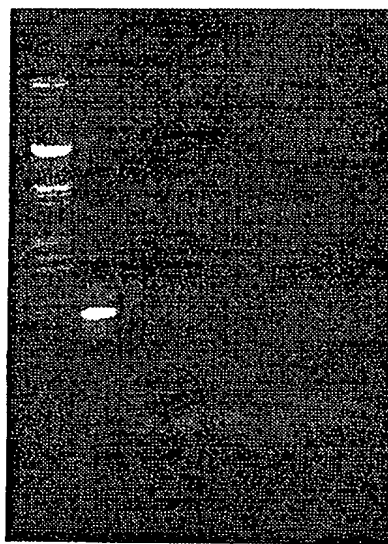


FIG. 4



1 2 3 4 5 6 7

**FIG. 5**

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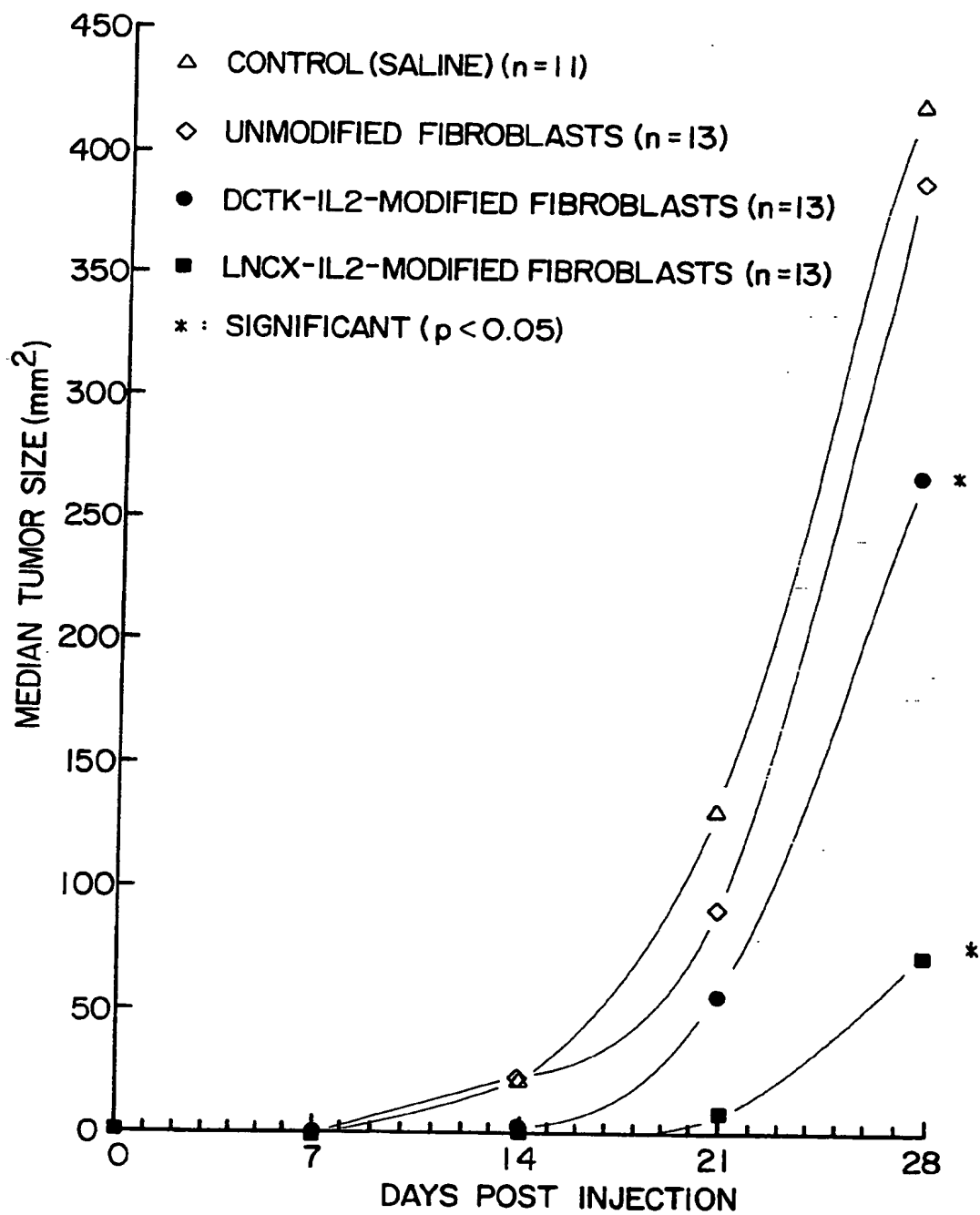


FIG. 6

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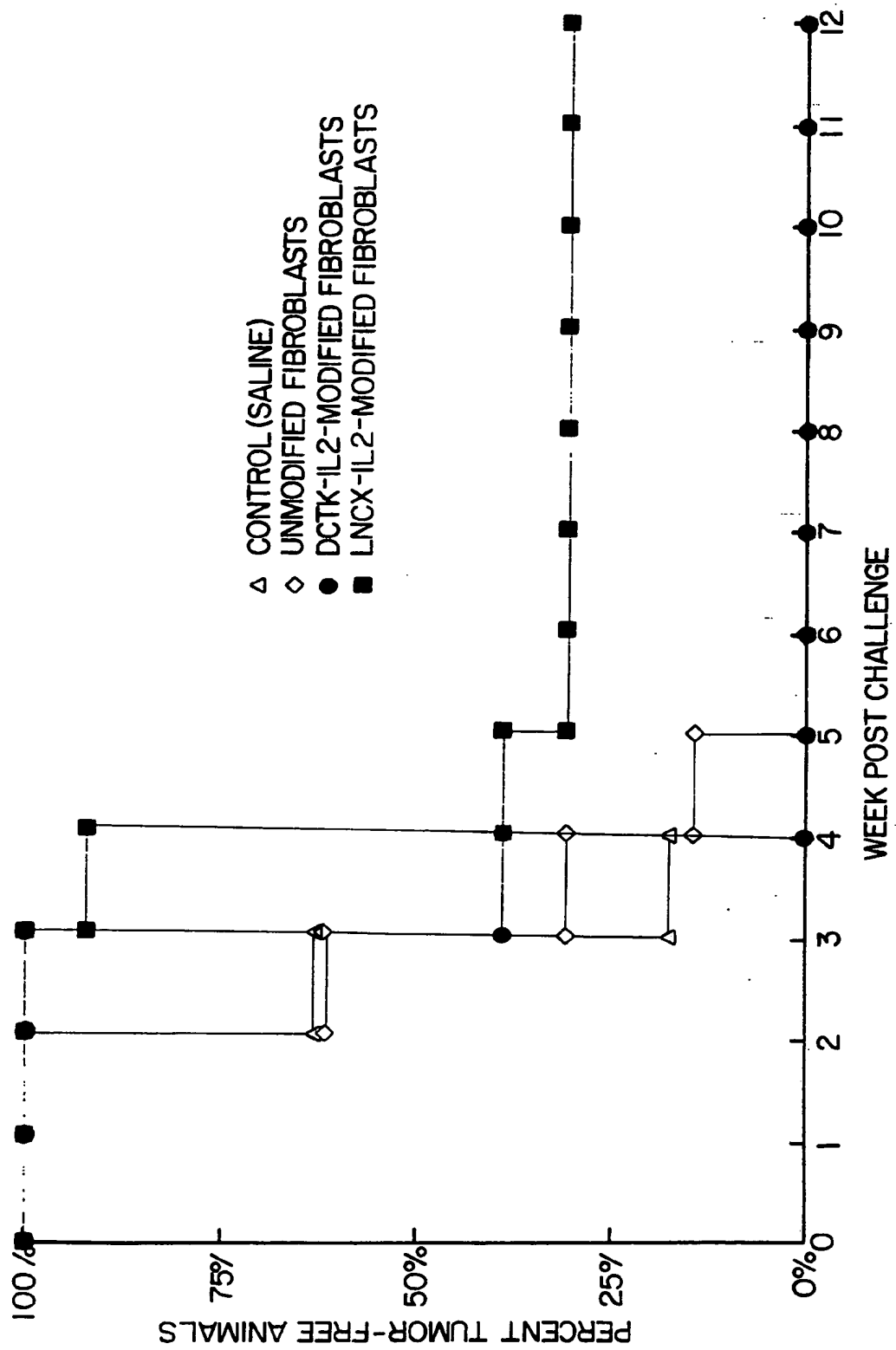


FIG. 7

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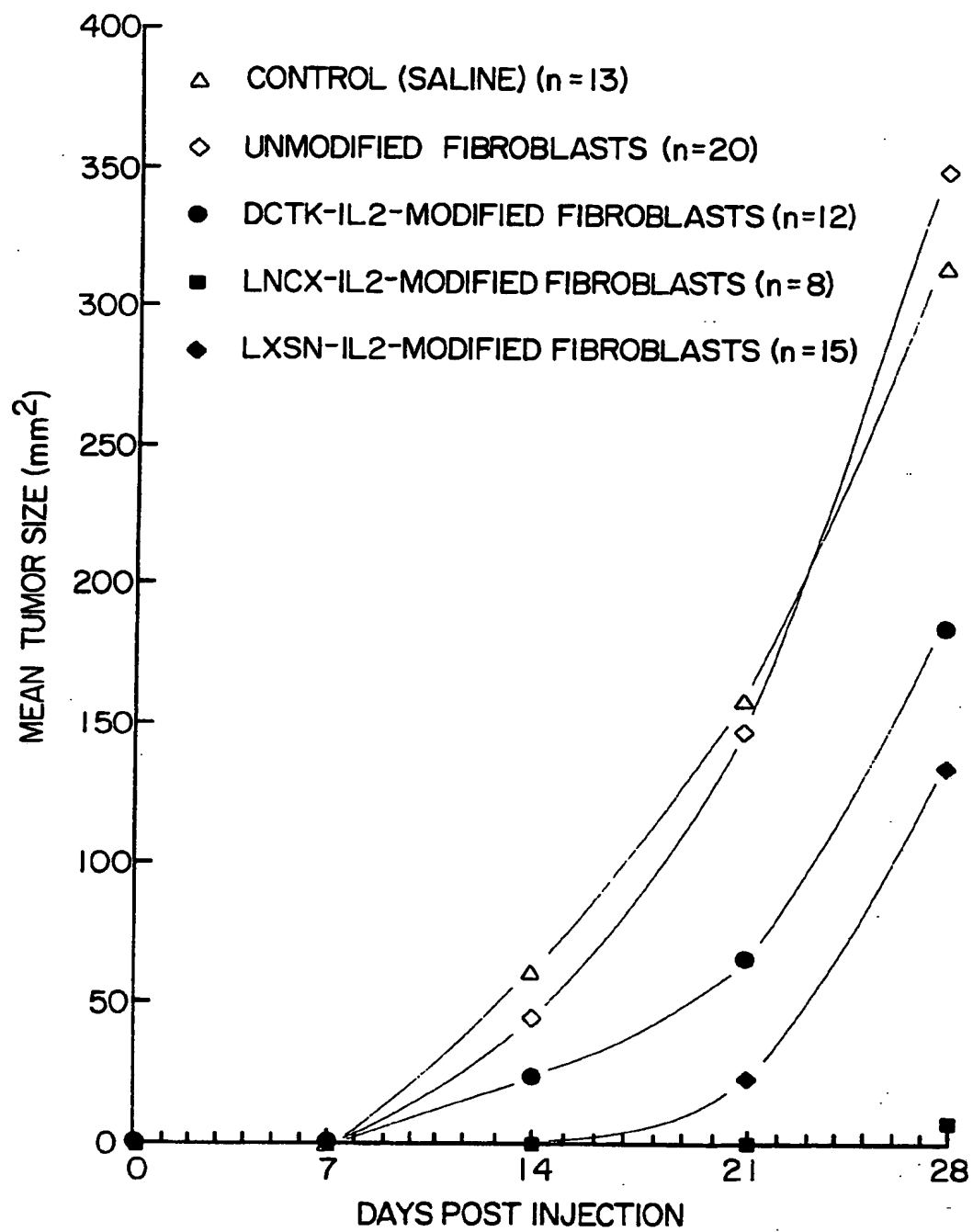


FIG. 8

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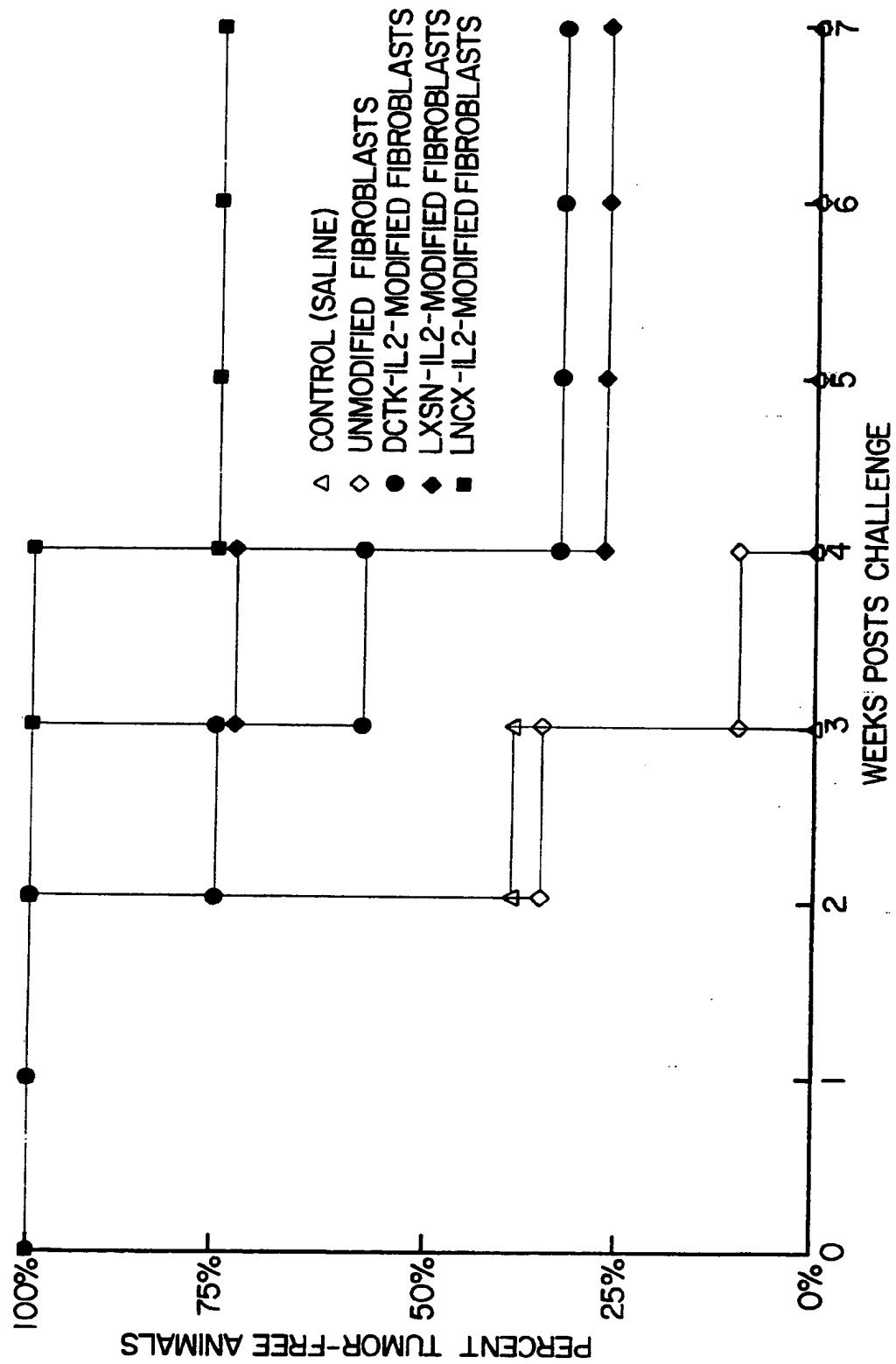


FIG. 9

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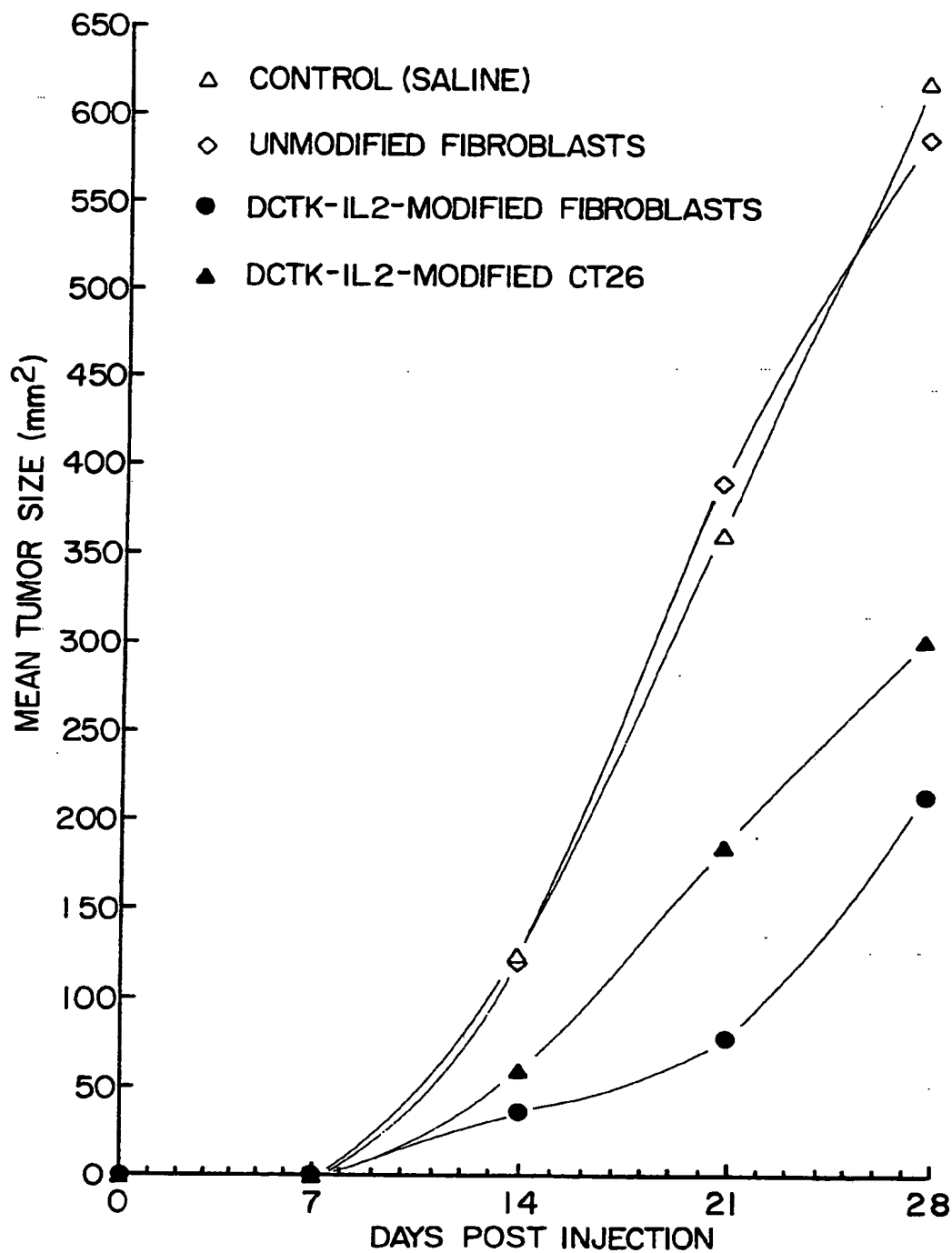


FIG. 10

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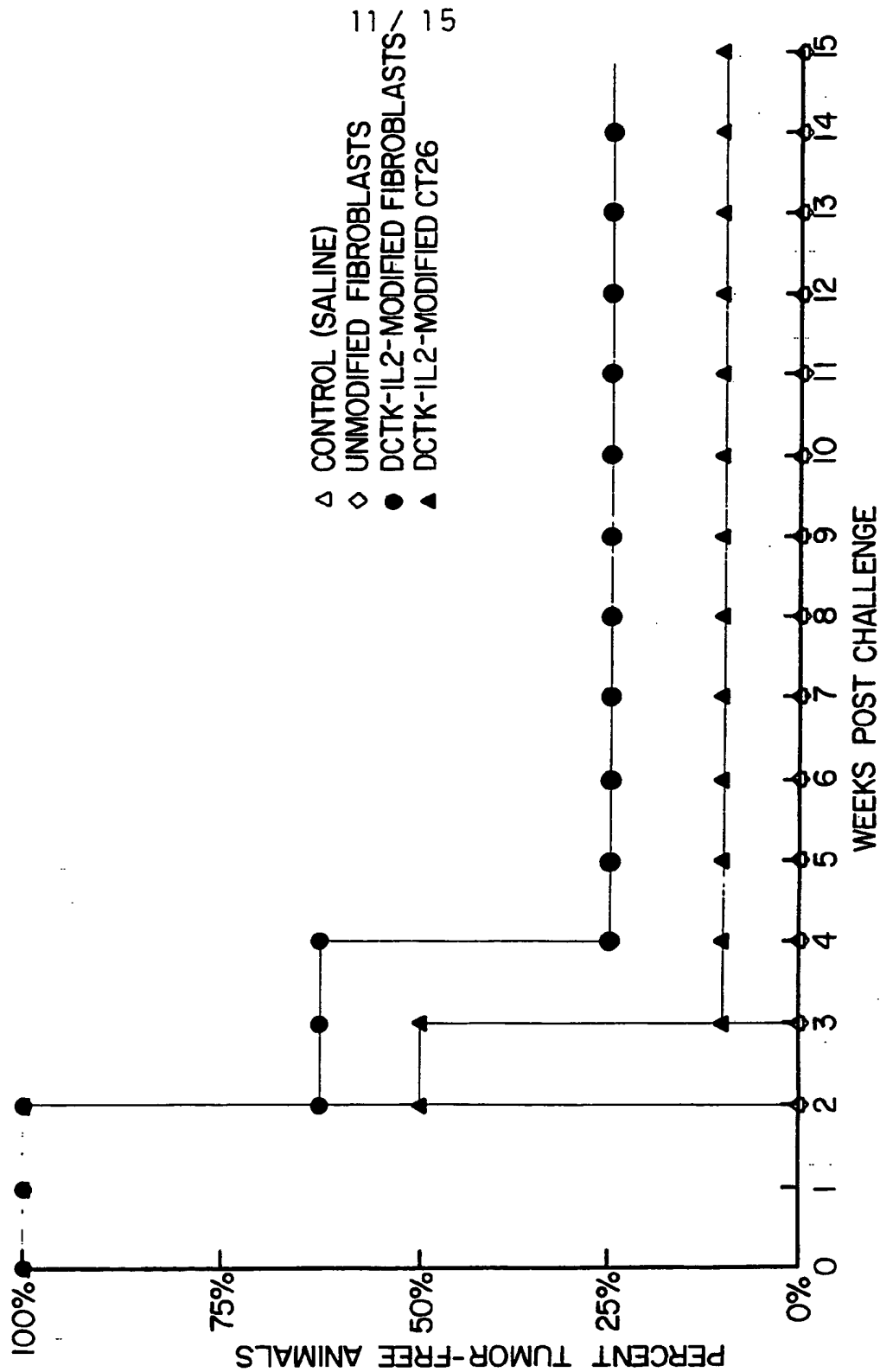


FIG. 11

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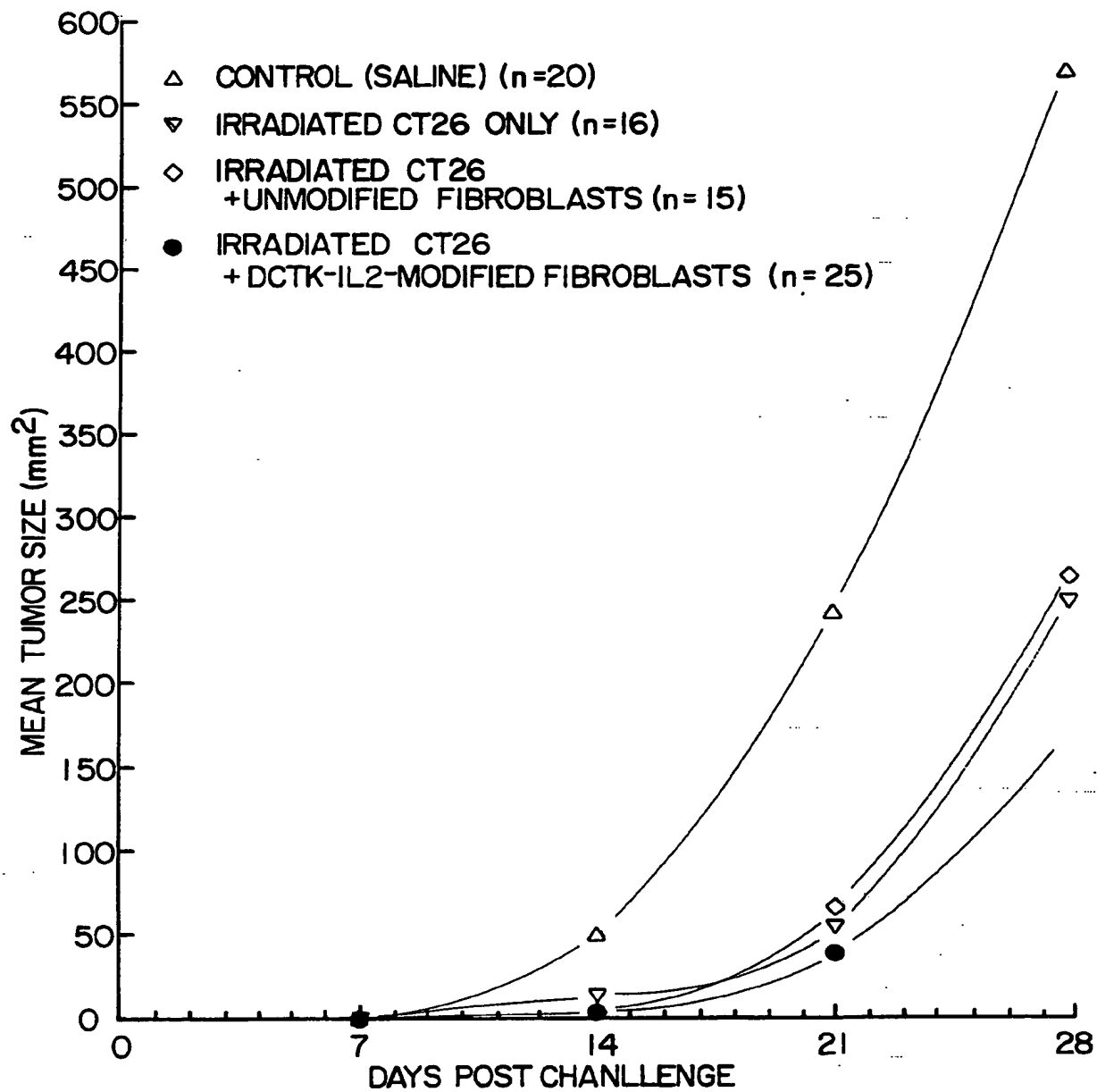


FIG. 12

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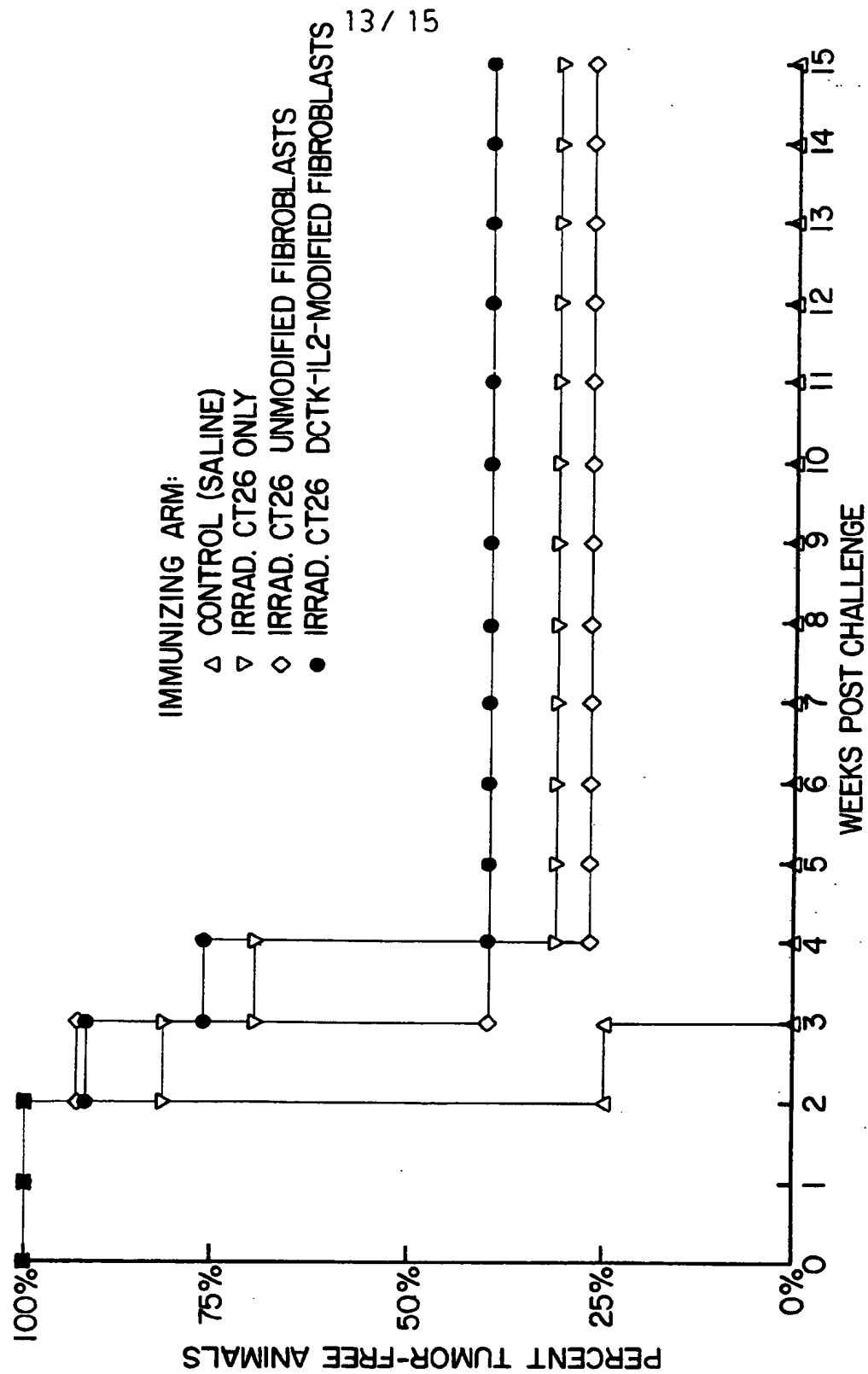


FIG. 13

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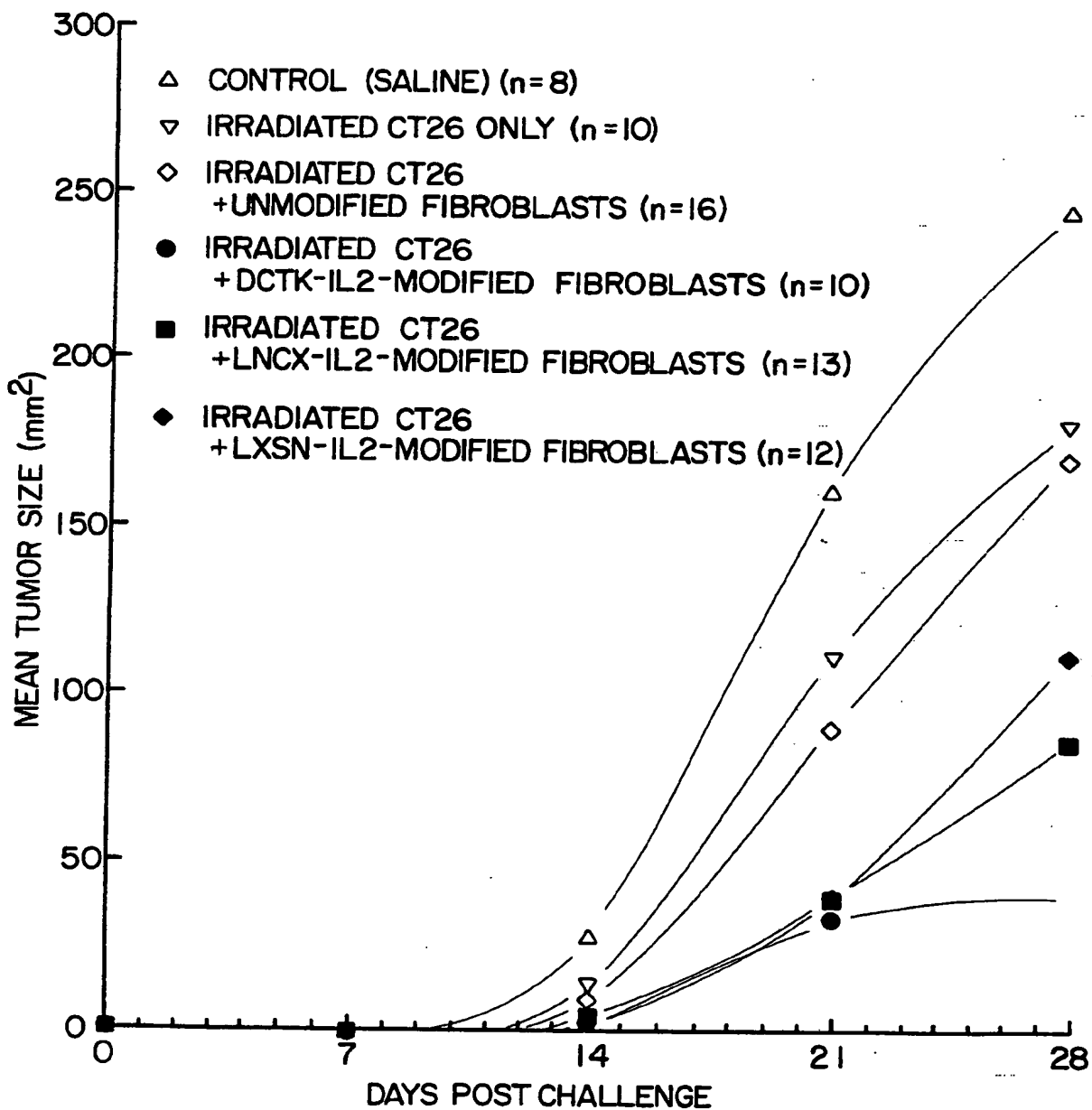


FIG. 14

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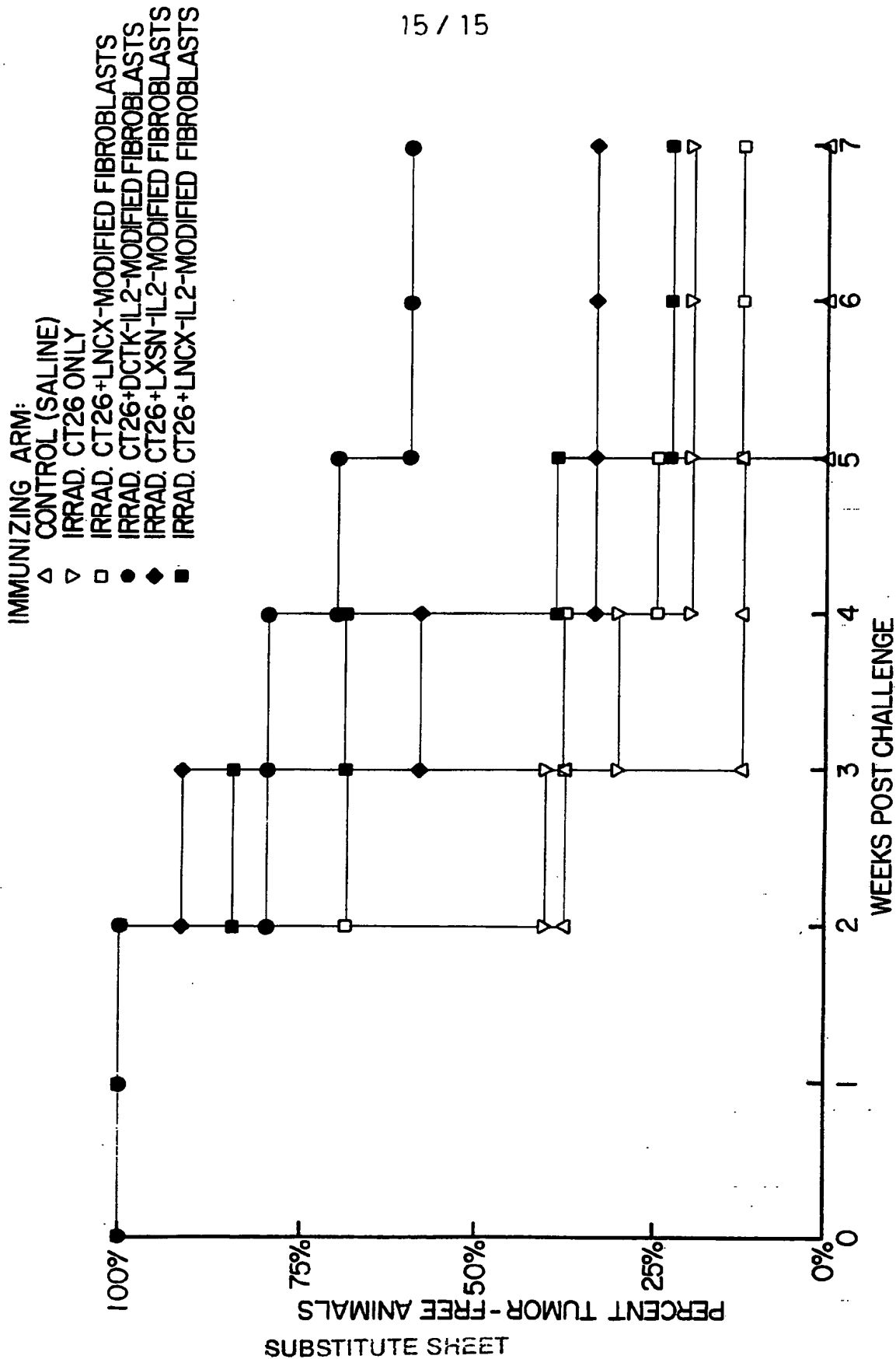


FIG. 15

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08999**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(S) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	Journal of Experimental Medicine, Volume 172, issued October 1990, Gansbacher et al, "Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity", pages 1217-1224, see the entire document.	<u>1-8, 11-14</u> 9, 10, 15, 16
<u>X</u> Y	Cell, Volume 57, issued 05 May 1989, Tepper et al, "Murine Interleukin-4 Displays Potent Anti-Tumor Activity In Vivo", pages 503-512, see the entire document.	<u>1-3, 5, 6, 8, 11, 12, 14</u> 4, 13
<u>X</u> Y	Cell, Volume 60, issued 09 February 1990, Fearon et al., "Interleukin-2 Production by Tumor Cells Bypasses T Helper Function in the Generation of an Antitumor Response", pages 397-403, see the entire document.	<u>1, 3-5, 8, 11-13</u> 2, 6, 7, 14-16
Y	Cancer Research, Volume 50, issued 15 August 1990, Ogura et al., "Implantation of Genetically Manipulated Fibroblasts into Mice as Antitumor $\alpha$ -Interferon Therapy", pages 5102-5106, see the entire document.	1-16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	g*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 January 1993	Date of mailing of the international search report 26 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JACQUELINE STONE
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08999

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Cancer Research, Volume 50, issued 15 December 1990, Gansbacher et al., "Retroviral Vector-mediated Interferon Gene Transfer into Tumor Cells Generates Potent and Long Lasting Antitumor Immunity", pages 7820-7825, see the entire document.	<u>1, 3, 5, 6, 8, 11, 12, 14</u> 2, 7

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08999

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 48/00, 35/12, 39/00; C12N 15/19, 15/24, 15/25, 15/26, 15/90, 15/63

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71